Serological characterization of autoantibodies in Autoimmune Haemolytic Anaemia and its clinical implications

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A study from tertiary care centre in South India

A dissertation submitted in partial fulfillment of
M.D. Immuno Haematology and Blood Transfusion Examination of the Tamil Nadu Dr M.G.R. UNIVERSITY, CHENNAI to be held in 2016

Certificate

This is to certify that the dissertation "**Serological characterization of autoantibodies in Autoimmune Haemolytic Anaemia and its clinical implications-A study from tertiary care centre in South India**" is a bonafide work of **Dr Rajeshwari B.** towards the M.D. (Immuno Haematology and Blood Transfusion) Examination of the Tamil Nadu Dr M.G.R. University, Chennai to be held in 2016.

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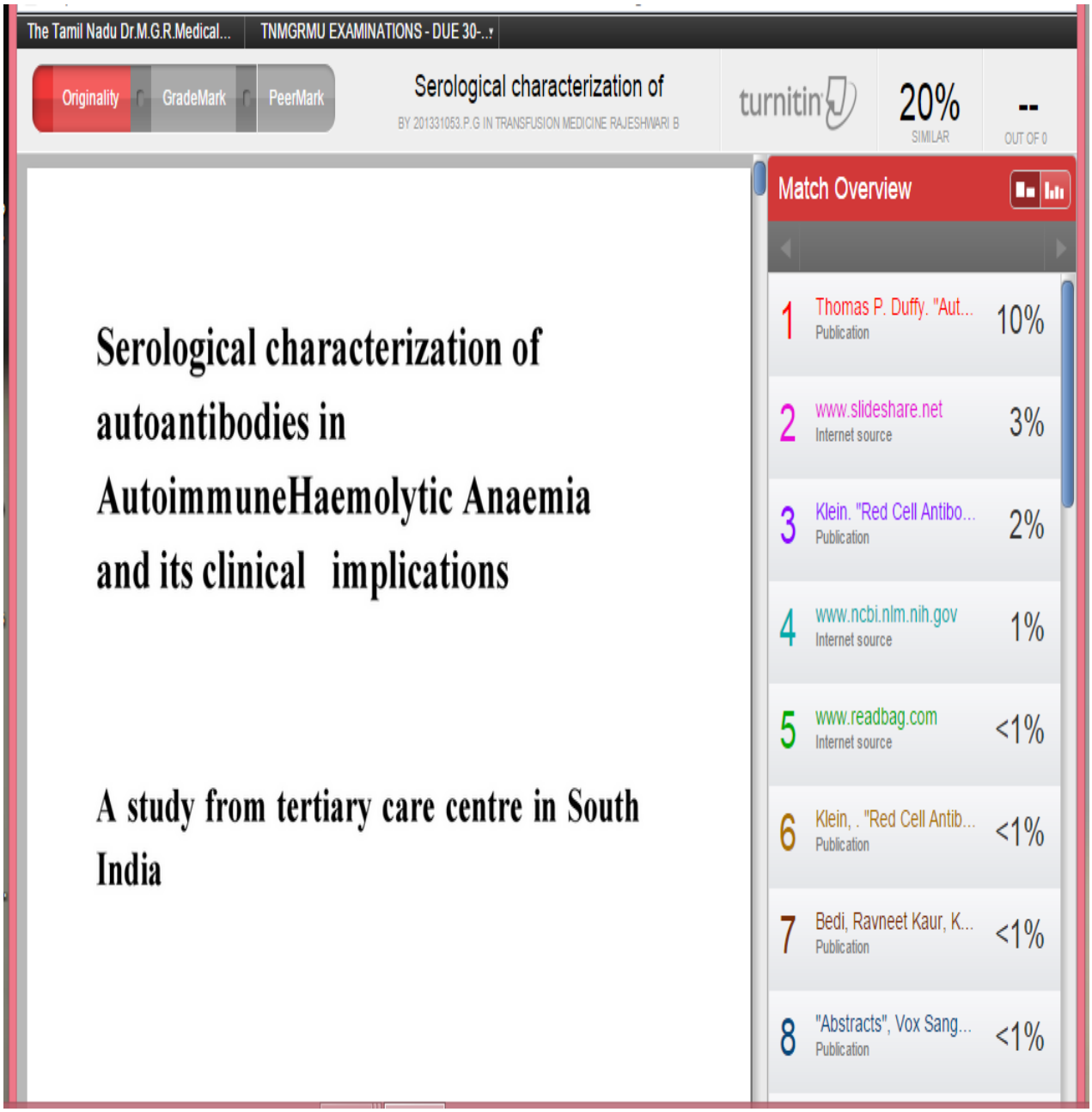
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ACKNOWLEDGEMENT

I wish to express my sincere heartfelt gratitude to my teacher, guide.advisor and above all a great human being Dr Dolly Daniel for her generous advice, inspiring guidance and encouragement throughout my course. This thesis would have not been possible without her continuous tireless professional input and encouragement. Her technique of explaining complicated subjects in simple way, keenness to understand new things, attention to detail and approachability is truly inspirational.I would also like to convey my sincere thanks to Dr Biju George for his advice and timely professional input when needed the most.

I would like extend my gratitude towards my teachers Dr Sukesh Chandran Nair and Dr Joy Mammen for their supervision, continuous effort to teaching and to improve the standards.

I would convey my sincere thanks to all the blood bank staff who helped me in collecting the data despite their busy schedule. I would like to acknowledge the enormous inputs given to this project by the biostatician Dr Visali and Ambily for professional input and support.

I also would like to express my whole hearted gratitude to my family for their support, encouragement, understanding and struggle to make me comfortable.

Lastly but not the least I must thank all the patients in the study who have allowed me to use their clinical and laboratory data for this project.

ABBREVIATIONS

AIHA—Auto immune haemolyic anaemia

DAT- Direct antiglobulin test

CAT- Column agglutination test

RBC-Red blood cells

Ig- Immunoglobulin

AHG-Anti human globulin

LDH-Lactate Dehydrogenase

SLE-Systemic lupus erythematous

CLL-Chronic lymphocytic leukaemia

PCH-Paroxysmal cold haemoglobinuria

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AIM of the study

To study the correlation between autoantibodies implicated in auto immune haemolytic anaemia (AIHA) and its relationship with *in vivo* haemolysis.

Primary objective of the study

To serologically characterize the type of autoantibodies resulting in AIHA and to correlate with *in vivo* haemolysis

Secondary objective of the study

To study the correlation between the strength of Direct Antiglobulin Test (DAT) and the severity of *in vivo* haemolysis.

INTRODUCTION

Autoimmune haemolytic anaemia (AIHA) is defined as decreased red cell survival or accelerated destruction, secondary to antibodies that are directed against the individual's own red blood cells. It is a relatively rare disease with varying clinical presentation.The disease is greatly heterogeneous, withsymptoms ranging fromfully compensated, to patients presenting with fulminant, rapid onset of life-threatening anaemia.

AIHA can be primary (idiopathic) or secondary. It affects all age groups, with the peak incidence of primary AIHA in the fourth and fifth decades. In secondary AIHA, age reflects the age distributionof the underlying disease. For example, in patients with SLE AIHA occurs in younger age group and usually in female population

AIHA is a heterogeneous disease, with respect to the type of the antibody involved and the presence or absence of an underlying condition resulting in AIHA. Majority of these autoantibodies react with high-incidence red cell antigens.These auto antibodies agglutinate, sensitize or cause lysis of red blood cells of their own as well as random donor red cells. Destruction of red cells causes anaemia, jaundice and without timely intervention it can be fatal.

Symptoms of AIHA can vary from mild anaemia to life threatening complications secondary to severe anaemia. So it is very important to identify patients with haemolytic anaemia so that these patients have timely intervention.

Autoimmune haemolytic anaemia can have a wide spectrum of clinical manifestations and should be suspected in a patient presenting with symptoms attributable to anaemia

(in the absence of obvious other causes like nutritional deficiency, bleeding etc)such as easy fatigability, shortness of breath,palpitations andassociated with jaundice. Occasionally massive haemolysis can occur which can manifest as severehaemoglobinemia and haemoglobinuria.

Patients with cold agglutinin disease may have history of haemolysis following cold exposure and may present with cyanosis of their distal extremities such as nose, ears and chin on cold exposure. Sky-blue mottling of the skin of the extremities called livedo reticulariscan occur as a result of agglutinated red cells obstructing the blood flow in the capillaries. Occasionally patients may also experienceRaynaud phenomenon.It is not uncommon to havea history of recent infection and antibiotic usage prior to onset of anaemia and jaundice.

Laboratory parameters which are helpful for the diagnosis of AIHA are

a. Complete blood count-
b. Reticulocyte count
c. Direct antiglobulin test(DAT)
d. Peripheral smear
e. Indirect hyperbilirubinaemia
f. Serum Lactate Dehydrogenase (LDH)

Direct coombs test (DAT) is an important serological test and helps in differentiating immunological with non immunological causes of AIHA. The diagnosis of AIHA normally depends on the demonstration of a positive DAT result, which indicates the presence of antibody and / or complement components or both on the surface of the

red cell. The DAT is done by addition of an antiglobulin to washed red cells, which leads to agglutination when the antibody, complement, or both are present on the red cell surface. A recommendedbroad-spectrum antihuman globulin reagent (polyspecific) contains antibodies for IgG immunoglobulin and complement components.

Once polyspecific DATis positive, DAT with monospecific antiglobulin is recommended to identify the subtype of AIHA, since the treatment differs with each subtype.

The severity of haemolysis can vary greatly leading to a wide spectrum of clinical presentation. Various studies in the literaturehave described a number of characteristic factors that has an impact on the severity of haemolysis. These factors include,

Antibody quantity, antibody specificity, thermal amplitude, ability to bind tissue macrophages and ability to fix complement. Additionally, characteristics of the target antigen which include the antigen density on the cell and its expression are also noted to have an impact on the severity of haemolysis.

The primary objective of this study is to assess the correlation between the presence of different types of antibody/ies and their clinical significance in terms of assessing the severity of *invivo* haemolysis. In this study polyspecific and monospecific DAT will be performed by column agglutination technique (CAT) to identify immunoglobulins and/or complement coated on the red cells. If patient has IgG antibody, then IgG subtyping will be carried out using the CAT technique in two dilutions.In most of the western studies IgG subclass was carried out using different platforms such as Enzyme linked immune sorbent assay (ELISA) and flow-cytometry. However

considering the cost and the need for batch testing, this is probably not a viable option at this time in our set up. The advantages of using Column agglutination technology (CAT) is that it is easily available, simple, robust and is a commonly used platform in immuno-haematology laboratories for various other tests. For example, blood grouping, antibody screening, antibody identification and red cell phenotyping.A study conducted by Dittamar et al. in the year 2001, comparing the efficacy of detection of DAT positivity on CAT vs.flow-cytometry showed that CAT was an equally sensitive platform to detect red cell bound antibodies(1). The CAT offers better sensitivity than other platforms used in immune haematology as demonstrated by Sudipta et al whose study was comparing CAT and 'tube technique' for patients of AIHA(2).

The secondary objective of this study is to assess whether the strength of the DAT correlates with the severity of the disease. In a study published by Wheeler et al(3), DAT strength of 2 + or more strongly correlated with haemolysis and a similar study fromWikaman et al also showed a strong correlation between the strength of DAT and severe haemolysis(4). Studies fromIndia correlating the strength of DAT with severity of haemolysis shows conflicting results. Study done in AIIMS by Choudhary et al did not find any correlation between DAT positivity and severity of anaemia(4)Currently the strength of DAT is not used for assessing the severity of haemolysis.If such a correlation exists, we can identify patients who are at high risk of haemolysis, and these patients can be kept under close surveillance.

There are many western studies which have looked in tovarious factors affecting the severity and correlation between DAT strength and *invivo* haemolysis. However there are very few studies from India regarding this and some of them have contradicting results. Considering AIHA can vary greatly in terms of aetiology, pathogenesis, clinical signs and symptoms it is very important to identify patients who are at high risk, so that management can be planned accordingly.

Despite better understanding of pathogenesis and modern laboratory approach, management of AIHA patients still remains a major challenge to clinicians and to blood banks.It is well known that trasnfusing AIHA patients can be challenging.This is because of the numerous difficulties encountered during ABO grouping and cross matching,. Hence specialized serological tests such as alloadsorption or autoadsorption are required.It is not uncommon that a fully matched donor is not found many a times to transfuse these patients.However, even in the absence of compatible blood, transfusion should not be withheld in a critically ill patient with life threatening anaemia. The "best match" or least "incompatible units" can be transfused to such patients under close supervision.

REVIEW OF LITERATURE

Autimmune haemolytic anaemia (AIHA) is a collective term for several diseases characterized by autoantibody-initiated destruction of red blood cells (RBCs).It is a rare disorder with wide variable manifestations. Patients can present to the physician with mild symptoms of anaemia or occasionally jaundice or rarely present with life threatening complications like myocardial infarction or cardiac failure secondary to severe anaemia.In view of wide variation in the clinical symptoms, it becomes very important to identify various factors which causes severe disease, so that patients who are at high risk of severe haemolysis are identified and appropriate therapeutic intervention initiated under close supervision.

HISTORY OF AIHA

Donath and Landsteiner were the first ones to describe autoimmune haemolytic disorder in the year 1904 on three patients of paroxysmal cold haemoglobinuria (PCH).The first experimental model of immunemediated haemolytic anaemia (IHA) was created by Dameshek and Schwartz in 1938 who induced an immune haemolytic anaemia by injecting heterologous red cell antibodies to guinea pigs. In 1943, Dacie and Mollisondemonstrated that patients with acquired haemolytic anaemia were noted to have an intrinsic factor (presumably an antibody) that resulted in increased red cell destruction.

The foundation of immunehaematology was laid by Coombs et al in 1945 by introduction of Coombs test (DAT or antiglobulin test), which permitted the

identification of the immune causesof AIHA. Use of DAT in patients with acquired haemolytic anaemia was done by Boorman et al and Loutit and Mollison in 1946.They demonstrated the importance of red cell autoantibodies in the pathogenesis of AIHA. Since then use of antiglobulin test/ Coombs test has allowed detection of different classes of immunoglobulin and presence of complement on the red cell membrane.This understanding of the disease pathophysiology has helped in the better treatment of AIHA patients.

EPIDEMIOLOGY AND AGE DISTRIBUTION

Autoimmune haemolytic anaemia is a rare disease. Based onpopulation studies, the incidence of AIHA is 0.8 to 1/ 80,000 to100 000/year in western population(6,7)and the reported prevalence is 17/100000.Frequency of this disorder is usually more common in females than in males. The male to female ratio is 40:60.Incidence and prevalence of AIHA from population based study from India are sparse.

Patients with AIHA with no identifiable underlying disease/cause are classified to have primary or idiopathic type. AIHA in patients with associated autoimmune disease and certainmalignant or infectious diseases as etiological causes are classified to have secondarytype. PrimaryAIHAaffects all age groups with the peak incidence in the fourth and fifth decades. Secondary AIHA reflects the age distribution of the underlying disease. For exampleLymphoproliferative disorders affects older age group, whereas autoimmune disorders like SLE involves youngerpatients. Women were noted to have a higher incidence ofboth idiopathic AIHA and secondary AIHA associated with SLE and other autoimmune disorders

ETIOLOGY OF AIHA.

The breakdown of immuneregulation has been implicated in the development of the autoantibodies. The loss of suppressor T-cell regulation of autoantibody production and the presence of overactive B cells lead to the emergence of autoantibodies (8). Many factors such as infections, drugs or inflammatory disorders, often serve asa potential trigger to initiate the process of autoantibody formation. Viral infections can initiate AIHA by greatly increasing the ability of macrophages to phagocytose erythrocytes which are coated with antibody(9).Occasionally, the autoantibody is directed against another target and because of cross-reactivity with the red cell antigens, red cell destruction occurs .

Secondary warm AIHA is associated with wide range of diseases such as Hodgkin and non-Hodgkin lymphoma, CLL, hairy cell leukemia, large granular lymphocytosis, Castleman disease, angioimmunoblastic lymphoma, immune thrombocytopenia, common variable immunodeficiencyetc. Warm type AIHA has also been documented with infections like subacute bacterial endocarditis. Inflammatory states such as ulcerative colitis and biliary cirrhosis also can manifest with warm-type AIHA. Rarely warm type AIHA has also been noted in patients who received Interferon-α treatment especially in large doses.

In literature many Familial cases of AIHA have been reported (10). Also more than 30 cases have been recorded in association with both benign and malignant ovarian neoplasms(11). AIHA also occurs after allogeneic stem cell transplantation(12) as

well as in patients with solid organ transplantation(13).Alloimmunization following transfusion may rarely be complicated by AIHA. Infection is a common antecedent to AIHA in children. The cold autoantibody which occurs with mycoplasma pneumonia infection has cross-antigenicity between the mycoplasma cell wall and the I antigen on the red cell membrane (14) and these autoantibodies are usually polyclonal IgM antibodies.

3. Classification of AIHA

Based onthe aetiology, AIHA is classified into primary (idiopathic) or secondary AIHA (16,17).The various causes resulting in secondary autoimmune haemolytic anaemia can be seen in the classification table1.

AIHA is classified into warm, cold and mixed type depending on the characteristic temperature reactivity of the autoantibody.

Warm autoantibodies are more reactive at 37ºC than at lower temperature. In case of cold-type autoantibodies, thesereactwith red cells morestrongly at 0ºC to 5ºC. These autoantibodies becomes clinically significant,only when their thermal range of reactivity extends to 28ºC - 31ºC or higher.Since this range of temperatureis encountered in the microvasculature of the skin, especially in the distal extremities, ears, and the tip of the nose haemolysis tends to be more marked here.

Incidence of different types of AIHA

Warm antibody AIHA accounts for approximately 75% of the cases (15), with an annual incidence of about 1 case per 75,000–80,000 population.Study done by Garraty et al on 347 patients noted warm type of antibody in70.3% of patients, cold agglutinin syndrome accounted for 15.6%, followed by drug-induced immune haemolytic anaemiain 12.4% and Paroxysmal cold haemoglobinuria in 1.7%(16). Altogether, the cold-reactive types accounts for approximately about 25% of all AIHA(15,17). Primary cold agglutinin disease(CAD) accounts for about 15% of all cases of AIHA(18).In several case series, mixed AIHA which has features of both warmand cold-type autoantibodies has been found in 6% to 8% of patients(19).

Thirdly immune haemolytic anaemia can occur secondary to drugs, this category is called drug induced AIHA which constitutes around 18% of AIHA(20)

TABLE 1 Classification of autoimmune haemolytic anaemia

Warm Autoantibody Type

- Idiopathic
- Secondary: lymphoma, chronic lymphocytic leukemia, hairy cell leukemia, systemic lupus erythematosus, other autoimmune disorders, ovarian tumors, chronic inflammatory disorders, following hematopoietic, cardiac, and solid organ transplantation

Cold Autoantibody Type

- Cold hemagglutinin disease
  - Idiopathic
  - Secondary
  - Transient (acute): *Mycoplasma pneumoniae* infection, infectious mononucleosis, other infections
  - Chronic: lymphoreticular malignancies
- Paroxysmal cold hemoglobinuria
  - Idiopathic
  - Secondary: viral and other infections, syphilis

Drug-Induced Immune Hemolytic Anemia

- Drug adsorption (hapten) type, eg, penicillin
- Drug-dependent antibody type (also referred to as immune complex), eg, second- or third-generation cephalosporins
- Autoimmune induction type, eg, α-methyldopa
- Nonimmunologic adsorption of protein, eg, cephalothin

Pathophysiology of AIHA

The pathophysiology of AIHA is complex. The process begins with opsonisation of the red cells by the autoantibody. A number of charactersitic factors that determine the degree of haemolysis has been described as early as 1970 byAbramson *et* al (21).Various factors which are related to the antibody including quantity of the antibody, specificity, thermal amplitude, ability to fix complement and ability to bind tissue macrophages have been implicated. Additionally, characteristics of the target antigen such as antigen density, its expression on the red cell and patient's age are also factors which influence the degree of haemolysis. Data published by Sokol *et al*.

in 1981 revealed that in the majority of patients with AIHA (80%) red cell destruction occurred extravascularly and involved red cells which were coated with antibody or complement or both, reacting with mononuclear phagocytes via specific receptors(22).

The charactersitics of both the target antigen as well as the bound antibody determine the degree of haemolysis. It is well documented that IgG antibodies are relatively poor activators of the classical complement pathway, but are easily recognized by the phagocytic cells.Extravascular haemolysis occurs in the reticuloendothelial system in the spleen and to a lesser degree in the liver.. On the other hand, IgM antibodies readily activate the classical complement pathway and produce cytolysis(21,23,24).

Warm agglutinin disease

In warm agglutinin disease or warm AIHA destruction of red cells occur by intravascular, extravascular, and cell-mediated mechanisms. Extravascular haemolysis occurs in the reticulo endothelial system i.e spleen and to a lesser degree, the liver. The immunoglobulinG (IgG)-coated red cells in warm type AIHA are sequestered primarily in the spleen. The IgG-coated red cells bind to macrophages byspecific membrane receptors for the Fc portion of the IgG, subclassesIgG1 and IgG3(25), and phagocytosis of the entire red cell by the macrophage sometimes follows. Most commonly, there is removal of only a small portion of the red cell membrane with creation of a microspherocyte released back into the circulation. These microspherocytes are altered cells, with a decreased surface area-to-volume ratio. These have a decreased life span because of their loss of plasticity and this leads to increased osmotic fragility of the red cell (26).

Cold agglutinin disease

There are two types of Cold haemagglutinin disease-one which occurs in a transient form and another which is chronic in nature. The transient form commonly occurs with infections such as mycoplasma pneumonia or infectious mononucleosis, primarily affecting adolescents or young adults. The antibody is usually IgM and polyclonal. Chronic cold haemagglutinin disease usually affects persons older than 50 years of age.In most of these patients there is no underlying illness identified; minority of them have lymphoproliferative disorders, including CLL, hairy cell leukaemia, lymphomas, and Waldenström macroglobulinemia.Cold agglutinins are most often reactive with the Ii blood groupsystem. The Ii antigens are carbohydrates closely related to theABO and Lewis blood groups and are present on red cells asglycoproteins and glycolipids(27).

Other specificities of cold haemagglutinins have been occasionally observed in patients with cold haemagglutinin disease, however identification of specificity is not indicated. The severity of haemolyisis that occurs in cold agglutinin disease depends on the titer and ability of the cold agglutinins to fix complement on red cells in vivo(28).

Patients who have cold agglutinins capable of reacting with red cells in the range of 28 to 31ºC will have ongoing haemolysis at ordinary room temperatures, whereas those patients with antibodies that react at a lower thermal range may have episodes of haemolysis only on cold exposure.

Complement mediated AIHA

Activation of the classic complement pathway is initiated even when a single molecule of IgM binds C1 (29).The C1 in turn activates C4 and C2, which binds tothe red cell and forms a C3 convertase enzyme complex. As the blood returns to the warmer temperatures within the body, cold agglutinins (29)convertase cleaves C3 to C3b and C3a. A single bound C3 convertase enzyme complex has the capability to cleave several hundred molecules of C3 to C3b, many of which bind to the red cell membrane. Circulating red cells coated with C3b and iC3b are trapped predominantly in the liver, where they are bound to the CR1 and CR3 complement receptors, respectively, on the membranes of hepatic macrophages.(29)This interaction of red cells with macrophages results in binding, sphering, andphagocytosis of red cells. The complement sequence goes to completion on some red cells, resulting in lytic destruction andintravascular haemolysis.

Paroxysmal Cold Haemoglobinuria

Paroxysmal cold haemoglobinuria (PCH) is a rare form of AIHA characterised by the sudden onset of severe haemolysis and is particularly noted to occur after an infection. This disease tends to be most common in children.

Pathophysiology of PCH:

The Donath Landsteinerautohaemolysin is an IgG autoantibody. This autoantibody reacts with red cells at reduced temperatures in the extremities and fixes theearlier components of complement.As blood circulates in the body, it returns to warmer temperatures where complement activation continues, causing some lysis in cells

coated with C3b. These cells are cleared by the liver and spleen.Whereas other cells coated with complement are lysed by the terminal components of complement. In most patients, the Donath-Landsteiner antibody is IgG with anti-P specificity(30).PCH is known to be associated with a wide variety of infections, including viral infections such as measles, mumps, cytomegalo virus, chickenpox etc and others such as mycoplasmal pneumonia, Haemophilus influenzae, Klebsiella pneumoniae, and Escherichia coli. Frequently, the cause of the preceding illness is not identified.

Drug Induced Immune Haemolytic Anaemia.

Several drugs are noted to result in a positive DAT result, however haemolytic anaemiaoccurs rarely. Four mechanisms by which drugs can cause IHA have been described(31).

a. Drug adsorption (hapten)- In the drug adsorption mechanism, antibodies which are induced by drugs react with a drug which is already firmly bound to the red cell membrane. Penicillin is a prototype of drugs that produce IHA by this mechanism. (32). Red cell damage mainly occursextravascularly, in the spleen by IgG auto antibodies . In this group of patients haemolysis is usually mild and the DAT shows IgG or occasionally IgG in combination with complement.

b. Immune complex(innocent bystander)

In this type of AIHA, drugs are known to bind transiently with proteins on the red cell membrane to form an immunogenthat stimulates production of an antibody(33) . The

antibody responsible may be IgG or IgM and activates complement. The DAT shows only C3d. Many a times the responsible drug is recognized by the antibody only when itis associated with a specific blood group antigen. The drugs which have been shown to react with cells rich in I antigen include Rifampicin, nitrofurantoin, and dexchlorpheniramine.(34,35)

c. Autoimmune Induction Mechanism-

The exact mechanism by which α-methyldopa induces AIHA is not known. Usually positive DAT result shows IgG and occasionally IgG along with complement.The incidence of haemolysis is rare in this group, and the degree of haemolysis is usually mild to moderateand patients were noted to develop anaemiagradually.

d. Membrane Modification (NonimmunologicProtein Adsorption)

In this category, it is hypothesized that certain drugs like cephalothin operate through the drug-adsorption mechanism and are able to modify RBCs so that plasma proteins like IgG, IgM, IgA, and complement can bind to the membrane. Since the uptake of immunoglobulin or complement is not the result of a specific antigen-antibody reaction, so this mechanism is non immunological. Haemolysis secondary to membrane modification is not observed so far.

DAT negative AIHA

This group was noted to show the same clinical features as DAT-positive AIHA, with the exception of an lesser amount of red blood cell bound immunoglobulin (Ig)G. Study done by 154 DAT negative AIHA patients by Toyomi Kamesaki et al showed that DAT−AIHA generally suffer milder anaemia and haemolysis with similar characteristics and response to therapy as that of DAT positive AIHA(36)

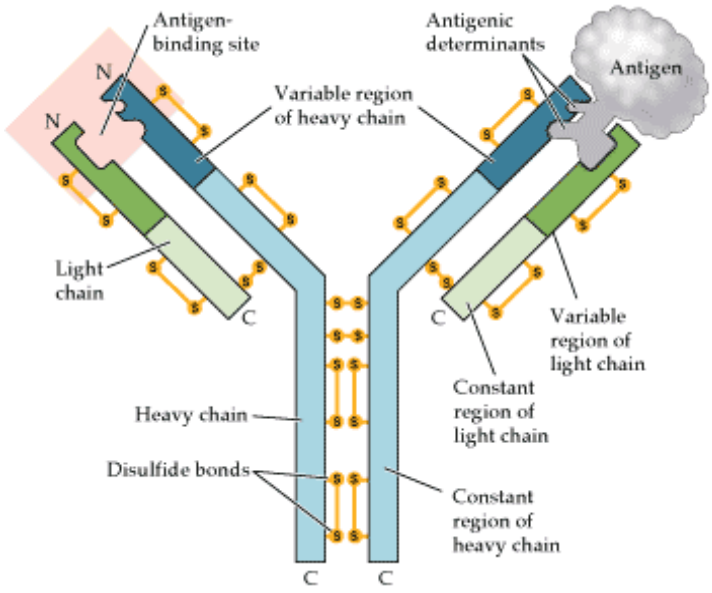
Structure of imunoglobulins: Immunoglobulin (Ig, is a complex protein, produced by plasma cells, with specificity towards specific antigens. That specific antigen stimulate the production of specific antibody.Theseantibodies are known to bind tothe antigen, fix complement, facilitate phagocytosis, and neutralise toxic substances found in the circulation. Thus, antibodies have various functions, some types of immunoglobulin are highly specialized and more specific than others.

Immunoglobulins are also called antibodies, theseare proteins, made up of four polypeptide chains- two identical heavy chains and two identical light chains. Light chains have molecular weight of approximately 22,500 Daltons andheavy chains have molecular weight of 50,000-75,000 Daltons, which are interconnected by covalent disulfide bonds. The heavy chains are held together by disulfide bonds at their hinge region. The H chains differ in structural and antigenic properties. These chains determine the class and subclass of the molecule.

There are total five types of heavy chain, these are gamma, alpha, mu, delta, and epsilon. The two types of light chains, named kappa (κ) and lambda (λ) respectively.

The light chains are the same in the five types of immunoglobulin molecules, it is thevariation in the heavy chain which make a difference in each type.

Depending on the structure of their heavy chains,immunoglobulins are classifiedinto five types,these are IgA (α [alpha] heavy chain), IgD (δ [delta] heavy chain), IgE (ε [epsilon] heavy chain), IgG (γ [gamma] heavy chain), and IgM (μ [mu] heavy chain). IgG is the most common and concentrated in serum, consisting of nearly 80% of the total serum immunoglobulin; second common immunoglobulin is IgA, at about 13%, this is predominant immunoglobulin found in body secretion,IgM is 6%; IgD is 1%; and IgE is the least common immunoglobulin, present at less than 1%.(37)



Structure of Immunoglobulin

Immunoglobulin are protein molecules have two terminal regions, these are the **amino** (-NH2) terminal and the **carboxyl** (-COOH) terminal.Amino terminal regions consists of both light and heavy chains of immunoglobulin's are known as the variableregions. This variation in the structure is according the great variation in

antibody specificity and this region is responsible for antigen binding.The carboxyl region of all heavy chains has a constant amino acid sequence and is named the constant region.The Fc region extends from the carboxyl terminal to the hinge region and is primarily responsible for monocyte binding and complement fixationon Fc receptors on cell. The Fab fragments extend from the amino terminal to the hinge region of the molecule.

Immunoglobulin molecule, domains are made up of regions of both heavy and light chains folded into globular structures or loops, and these are made up of approximately 110-120 amino acids. The domains consist of variable (V) and constant (C) regions made up of heavy and light chains. The number of domains is dependent on the immunoglobulin isotype. Three constant heavy chain regions (CH1, CH2, CH3) domains are noted on IgG ,Ig A ,IgD and four constant domains, CH1 to CH4, noted on the heavy chains of IgE and IgM . Particular biological properties of immunoglobulins, are associated with certain heavy chain domains especially with those of IgG and IgM, andinclude complement fixation.The hinge region of the immunoglobulin structure exists between the CH1 and CH2 domains of the heavy chain. Minor differences in these hinge regions are used to subtype IgG into four subclasses. In IgG molecules there is a specific constant heavy chain region (CH2 and CH3) which allow for attachment of Fc receptors of monocytes and macrophages.

IgM is a pentamer with a molecular weight of approximately 900 kDa and consists of five subunits of approximately 180 kDa each. Each subunit is linked by a J chain, which is a sulfhydryl-rich peptide (15 kDa), and it consists of two heavy chains μ and two light chains of type κ or λ. J chains contributes to the integrity and stability of the

pentamer structure of IgM. The Fc fragment of IgM is a cyclic pentamer with molecular weight of approximately 340 kDa.

There are two main forms of IgA. One is a monomer and the other exists in a polymer form—as dimers or trimers composed of two or three identical monomers, respectively, are joined by a J chain. IgA is located in different parts of the immune system, depending on subclass. In serum IgA is found in both monomeric and polymeric forms. Secretory IgA is normally found in the mucosal tissues of the body. The polymer form of secretory IgA acquires a glycoprotein secretory component when it passes through epithelial cell walls of mucosal tissues and this appears in nearly all body fluids. Another reason for the importance of IgA is that it can increase the effect of IgG induced RBC haemolysis.(38)**.**

IgE is normally found only in monomeric form in trace concentrations in serum, about 0.004% of total immunoglobulins, and is important in allergic reactions.

Sub type of IgG and haemolysis

IgG antibodies can be subdivided into four subclasses on the basis of minor structural differences in the hinge region of the IgG structure.The IgG subtypes are IgG1, IgG2, IgG3 and IgG4. The ratio of κ to λ in human IgG is 2:1, but the ratio is 1:1 and 8:1 for individual IgG2 and IgG4 subclasses respectively. The disulfide bonds linking the heavy chains also acts as factor for structural variation among the different subclasses. While IgG1 and IgG4 have two bonds each, IgG2 and IgG3 have four and five bonds respectively. The disulfide bond responsible for flexibility to the hinge

regions of the subclasses of IgG molecule and the distance or the angle Fab fragments form determines the antigen it can accommodate.(39)

Each of the subclasses exhibit differences in properties including placental transfer and complement fixation. While IgG1 and IgG3 bind to complement C1q molecule more strongly than IgG2. IgG4 doesn't bind at all and cannot activate the complement cascade. (40) . In case of IgG2, there are two alleles of the particular gene that encodes the FcRIIa receptor on macrophages. As a result, some people have a low-affinity receptor for IgG2 and these subjects shows, a positive DAT in the presence of an IgG2 autoantibody , without signs of haemolysis. Subject with high-affinity receptor and have potential to destroy IgG2-coated cells, however, IgG2-mediated haemolysis usually also depends upon antigen specificity.

Macrophages were noted to have IgG Fc receptors only for IgG1 and IgG3(25,41),therefore, the quantity and type of IgG on the red cell surface influences the degree of haemolysis. Studies done on IgG subtypes revealed that red cells coated with IgG1 alone or in combination with IgG2 or IgG4 require an average of 2000 molecules of IgG per red cell to stimulate phagocytosis and rosette formation in vitro. However in case of IgG3 subtype, an average of only 230 molecules of IgG3 per red cell are required for monocyte binding.(42). Only IgG1 and IgG3 are efficient in activating complement. Destruction of red cells is further enhanced when complement is also present on the red cell membrane.

Since two IgG molecules in close proximity are required to bind C1q and activate the complement system,(43) there must be a sufficient number of antibody molecules and antigenic sites for complement attachment. Once C1 is bound, C4 and C2 are activated to form C3 convertase, which then cleaves C3 and C3b. Several hundred molecules of C3b are bound to the red cell membrane through the action of a single C3 convertase enzymecomplex.(44)

The IgG Fc and complement receptors act together to enhance binding of red cells coated with both IgG and complement. Removal of IgG-coated red cells with or without complement occurs primarily in the spleen. Liver also participates in their clearance when large amounts of IgG are present on the red cell.(25).The spleen acts as a "fine" filter, the liver functions as a "coarse" filter, of sensitized red cells in AIHA.The most predominant subclass of IgG warm-type autoantibody is IgG1, which is found in most patients with warm-type AIHA.(45)**.**IgG3 seen in combination with other subclasses in 5%of patients as compared to solitary IgG3in only 3% of patients. (46)

AIHA has also been associated with IgA and IgM warm autoantibodies(47).AIHA caused by IgA antibodies alone in very rare, in this patients haemolysis occurring through similar mechanisms to those for IgG.(48).IgA coated red cells are removed from circulation by the same splenic process as are IgG-coated red cells. Receptors for IgA have been demonstrated on mononuclear phagocytes. Rarely IgA AIHA patients were observed to intravascular haemolysis, whichis mediated by the binding of C5b-9 complexes to bystander red cells independent of C3 activation.(33).

IgM autoantibodies have been shown to agglutinate or haemolyse normal red cells as well as enzyme-treated red cells and to sensitize red cells with or without fixing complement. Studies have shown that patients whose red cells are coated with IgA and IgM in addition to IgG are more likely to have lysis than those whose red cells are coated with IgG alone.(38,49)

It may not be possible to distinguish patients with haemolytic anaemia from those without haemolytic anaemia by the presnce or quantity of IgG3, but IgG3 is more commonly associated with haemolytic anaemia.(50) Study done by Dass et al noted that 75% of patients had haemolysis when red cells were coated with IgG1,IgG3 or both (49)

Immunoglobulin and complement activation-

The complement system is a complex group of more than 20 circulating and cell membrane proteins, with various functions within the immune response. Primary roles include direct lysis or destruction of cells, bacteria, and enveloped viruses as well as helping with opsonisation to facilitate phagocytosis

The complement proteins are activated by cascade of events mainly through three pathways:

- Classical,
- Alternative, and
- Lectin pathways.

The three pathways converge at the activation of the component C3. The classical pathway is activated by the binding of an antigen with an IgM, IgG1, or IgG3 antibody.The activation of the classical complement pathway is initiated when antibody binds to antigen. This allows the binding of the complement protein C1 to the FC fragment of an IgM, IgG1, or IgG3 subclass antibody. Ability of IgG antibody for complement activation depends on concentration of cell surface antigenand antigen clustering, in addition to antibody avidity and concentration. IgM is large and has FC monomers close to each other on one immunoglobulin molecule; therefore, only one IgM molecule is necessary to activate complement.

The C1 component is actually a complex composed of three C1 subunits, C1q, C1r, and C1s, which are stabilized by calcium .In the C1 complex bound to antigen antibody, C1q is responsible for catalyzing the C1r to generate activated C1s. Activated C1s is a serine-type protease. The C1qrs complex acts on C2 and C4 to form C4b2a. C4b2a uses component C3 as a natural substrate and C3b which is formed attaches to the microbial surface. Some of C3b binds to the C4b2a complex, and the resulting C4b2a3b complex functions as a C5 convertase. The C5 convertase then acts on C5 to produce C5a (a strong stimulator of anaphylatoxins) and C5b, which binds to the cell membrane and recruits C6, C7, C8, and C9 to the cell membrane. When C5b along with C6, C7, C8, and C9 are bound, the membrane attack complex forms; this causes cell lysis.

Clinical presentations- Signs and symptoms of AIHA is highly variable. Symptoms may vary from mild fatigue, shortness of breath and palpitationsto profound anaemia. Physical examination findings in AIHA are related to the severity of anaemia, which

include pallor, tachycardia, and mild jaundice; fever may or may not be present. The spleen is usually moderately enlarged. Patients with cold AIHA often present with symptoms of chronic anaemia. After cold exposure patients may present with episodes of acute haemolysis, which is manifested by haemoglobinemia andhaemoglobinuria. If the marrow is able to adequately compensate, the reduced red cell survival may not result in anaemia.

The diagnosis of haemolytic anaemia depends on clinical findings and laboratory results, such as haemoglobin or hematocrit values, reticulocyte count bilirubin, haptoglobin, LDH levels,red cell morphology and sometimes, red cell survival studies. The serologic findings help determine whether the haemolysis has an immune basis and, if so, what type of immune haemolytic anaemia is present. This is important because the treatment for each type is different.

In some cases, the destruction of red cells takes place in the intravascular space with the release of free haemoglobin into the plasma. The red cells are ruptured following activation of the classical complement cascade. Conversely and more commonly, extravascular haemolysis results when macrophages in the spleen and liver phagocytose red cells completely or partially (producing spherocytes) or destroy red cells by cytotoxic events, resulting in an increase in serum bilirubin.

Patients with cold agglutinin disease often present with symptoms of chronic anaemia. They may also manifest with haemoglobinemia and haemoglobinuria after cold exposure, causing acute haemolysis.Patients may present withcyanosis of their distal extremities, nose, ears, and chin on cold exposure. A sky-blue mottling of the skin of

the extremities called livedo reticularis is a result of agglutinated red cells obstructing blood flow in the capillary bed. Some patients may experience Raynaud phenomenon. Rarely, a patient develops a vascular occlusion, usually of a distal extremity, which is precipitated by prolonged exposure to cold. Patients with IgA monoclonal cold antibody manifest acrocyanosis but no haemolysis, because IgA does not activate the classic complement pathway.

Patients with mixed AIHA tend to have severe haemolysis compared to other types of AIHA. Most commonly mixed type is associated with lymphoma or SLE.

Factors affecting the severity of AIHA.

Quantity and class and subclass of immunoglobulins- Study done by Stratton et al showed that subjects whose red cells are coated with less than 950 IgG1 molecules per cell showed positive DAT but no signs haemolysis. On the other hand in patients with 1200 or more the number of more IgG1 molecules per cell was found to have haemolysis(51) This finding was consistent with the observation that at least 1180IgG1 molecules must be bound per cell for adherence to monocyte receptors to occur *in vitro*(52).Study done by van der Meulen et al showed clear relationshipbetween the theseverity of haemolytic anaemia and number of IgG1 molecules per cell.

The role of IgG2 autoantibodies is not clearin subjects with high-affinity FcRIIa receptors, alloantibodies with A specificity could cause haemolysis but those with Rhspecificity are not (Kumpel *et al*. 1996). IgG3 antibodies can cause lysis by

monocytes even when present on red cells at too low a concentration to be detected in the normal DAT, which explains why the test is negative in some patients with AIHA.A study done in China Li Z et al showed IgG3 positive patients had the most severe AIHA(53). IgG4 autoantibodies do not cause red cell destruction.

Complement activation

The ability of different IgG subclasses to cause lysisof red cells is also related to the nature of their interactionwith Fc receptors and their ability to activate complement.The IgG Fc receptor family consists of several activating receptors and a single inhibitory receptor.Four activating receptors (FcγRI, FcγRIIIaFcγRIIa, FcγRIIIb and one inhibitory receptor (FcγRIIb) is known in human beings.

Multiple antibodies and antigen specificity and its effect on haemolysis

Studies from literature shows that the presence of multiple autoantibodies or more than one immunoglobulin class on the red cells is associated with severe haemolytic anaemia.(54,55)Garratty et al in 1997 described three severe cases in which 2 were fatal and one case of severe AIHA associated with warm IgM autoantibodies and their specificities of each antibody (Ena, Wrb and Pr) are all associated with glycophorin A. The severity of AIHA caused byantibodies of these specificities may be related to the role of glycophorin A an inhibitor of red cell lysis by complement (Tomita *et al*. 1993). A few warm autoantibodies are specific for one particular antigen such as e or D or c or LW or band 3, or band 3 and glycophorin A and various other antigens.

Functional ability of reticuloenthelial system- Sensitized RBCs are phagocytized by interaction with RES mononuclear phagocytes, depending on which protein coats the erythrocytes. If only IgG coats the RBCs, gradual phagocytosis of the erythrocytes occurs. If both IgG and C3b coat theRBCs, there is a rapid phagocytosis, because the C3b fragment augments the action of IgG, enhancing sequestration and phagocytosis of the coated erythrocytes. If only C3b coats the RBCs, transient immune adherence occurs. It has been estimated that more than 100,000 molecules of the complementfragment would be required to induce phagocytosis, therefore, the activity of the macrophages and the severity of haemolysis via phagocytosis of sensitized RBCs depend on various factors influence macrophage activity.

Laboratory parameters.

Anaemia- warm autoimmune anaemia presents with varying degree of severity of anaemia. In case of warm AIHA significant patients will have haemoglobin less than 7gm/dl ,might need red cell transfusion, however in case of cold agglutinin disease anaemia is usually of moderate severity and is mainly due to extravascular haemolysis. In case of mixed autoimmune haemolytic anaemia, haemolytic anaemia may be more severe and may also be intravascular.

Reticulocyte count – Reticulocyte count is most often elevated in response to the patient's anaemia. Occasionally reticulocytopenia or increase in reticulocytes less than one would expect for the degree of anaemia in haemolytic patients, suggests the presence of immune-mediated destruction or apoptosis of red cell precursors, marrow suppression (eg, infection, malignancy, chemotherapy), or concomitant nutrient

deficiency n particular. Reticulocytopenia in a patient with severe anaemia appears to be a feature indicating a worse prognosis, (56,57)

Serum levels of lactate dehydrogenase/ LDH-Majority of patients with haemolysis were noted to have high levels of LDH, In study done by Roumier M et al noted, elevated LDH in 93 percent of patients with AIHA(58)

Unconjugated /Indirect Bilirubin -Serum levels of indirect bilirubinwere elevated in 87 percent of patients in one series(58), with median levels in the range 2.0 to 3.0 mg/dL (59).

Direct antiglobulin test (DAT)/Coombs test – Even in the presence of valuable clinical clues that may suggest a specific diagnosis, the definitive confirmation of the type of autoimmune haemolytic anaemia depends on the laboratory. DAT is a simple serological test, which is used to determine if red cells have been coated in vivo with immunoglobulin (Ig), complement, or both. A positive DAT result may or may not be associated with immune-mediated haemolysis.(60) DAT should be done in every patient with haemolysis to differentiate immune causes from non immune causes of haemolysis. The predictive value of a positive DAT result is 83% in a patient with haemolytic anaemia, but only 1.4% in a patient without, haemolytic anaemia (36). Depending on the technique and reagents used, the DAT can detect 100 to 500 molecules of IgG /red cell and 400 to 1100 molecules of C3d/red cell. However, the DAT result can be positive, coincidentally, in patients with haemolytic anaemia that is not immune mediated. Conversely, some patients with immune

haemolytic anaemia have a negative DAT result. The interpretation of a positive DAT result should take into consideration the patient's history, clinical data, and results of other laboratory tests.

Complement levels — In patients with cold agglutinin disease, serum levels of complement proteins C3 and C4 are low in most patients with cold agglutinin disease because of constant consumption, which may limit further extra- and intravascular haemolysis (61)

A peripheral blood smear –In case of extra vascular haemolysis peripheral blood smear usually shows the presence of spherocytosis , although spherocytes might not be obvious in milder cases (58). Red blood cell indices may show an elevated mean corpuscular haemoglobin concentration (MCHC), consistent with the presence of spherocytes and/or an increase in the mean corpuscular volume (MCV) indicative of an increase in the percent of reticulocytes. In case of cold agglutinin disease red blood cell aggregates will be evident on smear.Clumping of red cells can leads to error in calculating red cell indices due to autoagglutination. An elevated mean corpuscular volume, which corrects on heating the blood sample is good evidence of a cold agglutinin disease.

Bone marrow — Bone marrow usually shows erythroid hyperplasia and may show the presence of lymphoplasmacyticaggregates. The latter are often nearly monoclonal on analysis and consist of cells that are making the antibody. Aggregates may become larger and confluent with development of lymphoma .

Cold agglutinin titration — Blood sample must be collected and maintained at 37 to 40°C until the clot has formed and retracted and the serum has been removed.The titer of cold agglutinin in the serum is highly variable among affected patients,usually the titre is more than 1:2000.All normal individuals in low titer of cold agglutinins,(less than 1 in 64, and usually less than 1 in 10), with no age or seasonal variation (62). Haemolysis is rarely seen if the titer is less than 1 in 512, although exceptions exist(63).

Serum haptoglobin levels were reduced in 93 percent of patients, often to not measurable levels (15)

Haemoglobinemia haemoglobinuria and urine haemosiderin is often present in subjects with severe autoimmune haemolytic anaemia.

Treatment of AIHA.

Warm AIHA- In children, AIHA is usually a self-limited disease, haemolysis occurs after 2 to3 weeks after viral infection and settling down within 2 to 3 months. In case of adults is usually chronic and may be variably manifest for months to years.

Transfusion-The decision to transfuse AIHA patients is mainly dependenton the need to increase the oxygen-carrying capacity of the blood. Transfusion of red cells should be considered for patients, who are at risk of life-threatening anaemia ,underlying heart disease or cerebrovascular ischemia. In patients with signs and symptoms of severe anaemia immediate transfusion, can be life saving. Cross matching blood can be a major problem while planning transfusion to these patients, since the autoantibody may result in a positive antibody screening test and an incompatible

cross match. Most compatible blood should be given slowly to patient under close observation for haemolysis conversely, no patients houldbe deprived of transfusions when severe anaemia complicates AIHA.

Glucocorticoids-First line therapy is short-acting glucocorticoids, majority of patients show marked improvements, with reduced haemolysis and stabilization, followed by an increase in haematocrit.

Several mechanisms have been proposed for the beneficial effect of prednisone. Its most important, immediate action is to decrease red cell haemolysis by interfering with monocyte-red cell interactionsin the spleen and liver. A later action is a reduction of theautoantibody production. Prednisone also may act by decreasing the binding affinity of the autoantibody for red cell antigens.

Second-line agents for poorly responsive or relapsed disease —Patients not responding to initial treatment with glucocorticoids, those relapsing or relapsed following their discontinuation, and/or those who require high doses of glucocorticoids to maintain response. There are a number of agents available Riuximab, danazol, splenectomy, immunosuppressive agents.

Splenectomy-In warm AIHA, splenectomy removes the major site of red cell destruction as well as a site for antibody production. Based on numerous case reports, approximately 60% of patients will respond completely or partially to splenectomy.

Riuximab -The monoclonal CD20 antibody, a chimeric monoclonal antibody directed against the B cell surface protein CD20. It is thought to eliminate B cells via apoptosis, antibody-dependent cytotoxicity, and complement-mediated lysis. It used

as a second line drug in AIHA, ITP and EVANS syndrome and various other autoimmune disorders.

Immunosuppressive Therapy- Azathioprine, cyclophosphamide , and chlorambucil are the cytotoxic drugs which are used in AIHA.

Otherdrug which are used are danazol, cyclosporine, mycophenolate, antilymphocyte or antithymocyte globulin with variable success.

Treatment of cold agglutinin disease. -Patients with cold agglutinin disease usually presents with mild anaemia and treatment is given only if they are symptomatic. Patients are advised to keep warm particularly extremities. In cases of severe haemolytic anaemia glucocorticoids and splenectomy generally are not effective unlike in warm AIHA, chlorambucil or cyclophosphamide is used and this results in decreased titres of cold agglutinins and reduced haemolysis.

α-Interferon therapy has been given to patients with severe cold agglutinin disease with variable results. CD20 monoclonal antibodies have been successful in treatment of patients with cold agglutinin diseaseFludarabine-rituximab combination therapy is very effective, with a response rate of 75% in chronic cold agglutinin disease(61).

In this study we would like to serologically characterize the autoantibody and grade thestrength of the DAT reaction and correlate with clinical severity of AIHA. According to Dacie et al, in a historical review of AIHA in the year 2011.The characterization of autoantibodyin the serum helps to differentiate various types of AIHA and this gives a better assessment to the clinician regarding the likely course of

disease and helps to decide the form of treatment to be given. Additionally, IgG subclass determination, will also help in predicting the prognosis of the disease'(64). From the various studies in literature, it is known that IgG is the most common immunoglobulin associated with AIHA. Amongst them IgG1 subtype is the most common followed by IgG3 (in combination with IgG1 or alone) and have strong potential to cause haemolysis. It has also been suggested that presence of multiple antibodies on red cell membrane causes severe haemolysis, compared to single antibody coating the red cell membrane. Very few studies are availablein the literature regarding characterization of antibodies in AIHA and utilizing these parameters for patients' day to day management. Most of the western studies used either enzyme linked DAT or flow cytometry to assess presence of immunoglobulin adhere to red cells. There arevery few Indian studies on AIHA looking at the various factors influencing severity of AIHA by using column agglutination technique.There is also little data on the utility of the strength of DAT test as a marker for assessing the severity of haemolysis.In this study we propose to characterize the presence of particular immunoglobulin/sand /or complement and also grade the strength of DAT by column agglutination technique and correlate these parameters with the severity of AIHA.

If this studyshows correlation between and strength ofDAT with clinical severity, this may help in selecting the patients who need closesupervision during treatment and possible treatment intensification at an earlier stage. This may also help the clinicians in deciding the extent to which patients need to be followed upfor haemolysis and in prognostication.

MATERIAL AND METHODS

SETTING:

1. This cross sectional study was carried out in the Departments of Transfusion Medicine and Immunohematology (Blood bank) and department of Haematologyat Christian Medical College hospital, Vellore, Tamil Nadu. It is a 3000 bedded teaching hospital, providing tertiary medical care to the residents of Vellore and surrounding districts of Tamil Nadu, Andhra Pradesh, Karnataka and Kerala. It also serves as a referral centre for patients fromrest of India and South East Asia.

2. In this study, patients with DAT positive results and who also had clinical and laboratory evidence of haemolysis were included. The study was done over a period of one year, spanning between, May2014 – June 2015.

3. The primary objective of this study was to serologically characterize the type /subtype of auotantibodies /complement resulting in AIHA and correlate their presence with severity of *invivo* haemolysis

4. The secondary objective of this study was to correlate strength of the DAT with the severity of haemolysis.

5. In this study, column agglutination technique(CAT) was used to serologically characterize the autoantibodies. PolyspecificDAT was performed usingLISS Coombs ID card, to identify the presence of IgG and complement. Further testing was done

using monospecific DAT using LISS Coombs ID card 'IgG,IgM,IgA,C3d,C3c ,to identify the presence of immunoglobulins such as IgG,IgA,IgM and complement C3d,C3c.If patients were noted to have monospecific IgG , then further subtyping of IgG was done using LISS coombsID card "DAT IgG1/IgG3" (BIO-RAD) in 1:1 and 1:100 dilution.

6. Severity of haemolysis was classified into severe or moderate based on studycriteria laid down by Dass et al.(55)

METHODOLOGY

PARTICIPANTS: All patients who had DAT positive results betweenMay 2014 to June2015 were evaluated for study eligibility.

Inclusion criteria

Patients, who were positive for polyspecific DAT and noted to have haemolysis, based on clinical and laboratory criteria were enrolled in the study.

Time of presentation-

Patients enrolled belonged to different categories. These patients are

- First time diagnosed to have AIHA.
- Patients already diagnosed to have AIHA and on treatment at the time of analysis
- Previously diagnosed to have AIHA, currently relapsed after stopping treatment

All these category of patients were included in the study.

Severity of haemolysis

Haemolysis was assessed based on the laboratory parameters, which were noted from medical records or from clinical works station.The laboratory parameters used to categorise severity of haemolysis are

1) Haemoglobin <9gm/dl

2) Bilirubin (>2mg/dl)

3) Reticulocytes (>2%) and

4) LDH>500IU/ml.

Haemolysis was classified into severe if all the above parameters were fulfilled, or classified into moderate on the basis of whether two or three of the above laboratory parameters mentioned above are abnormal (3,55,65).

Exclusion Criteria

1) Patients who had haemopoietic stem cell transplant

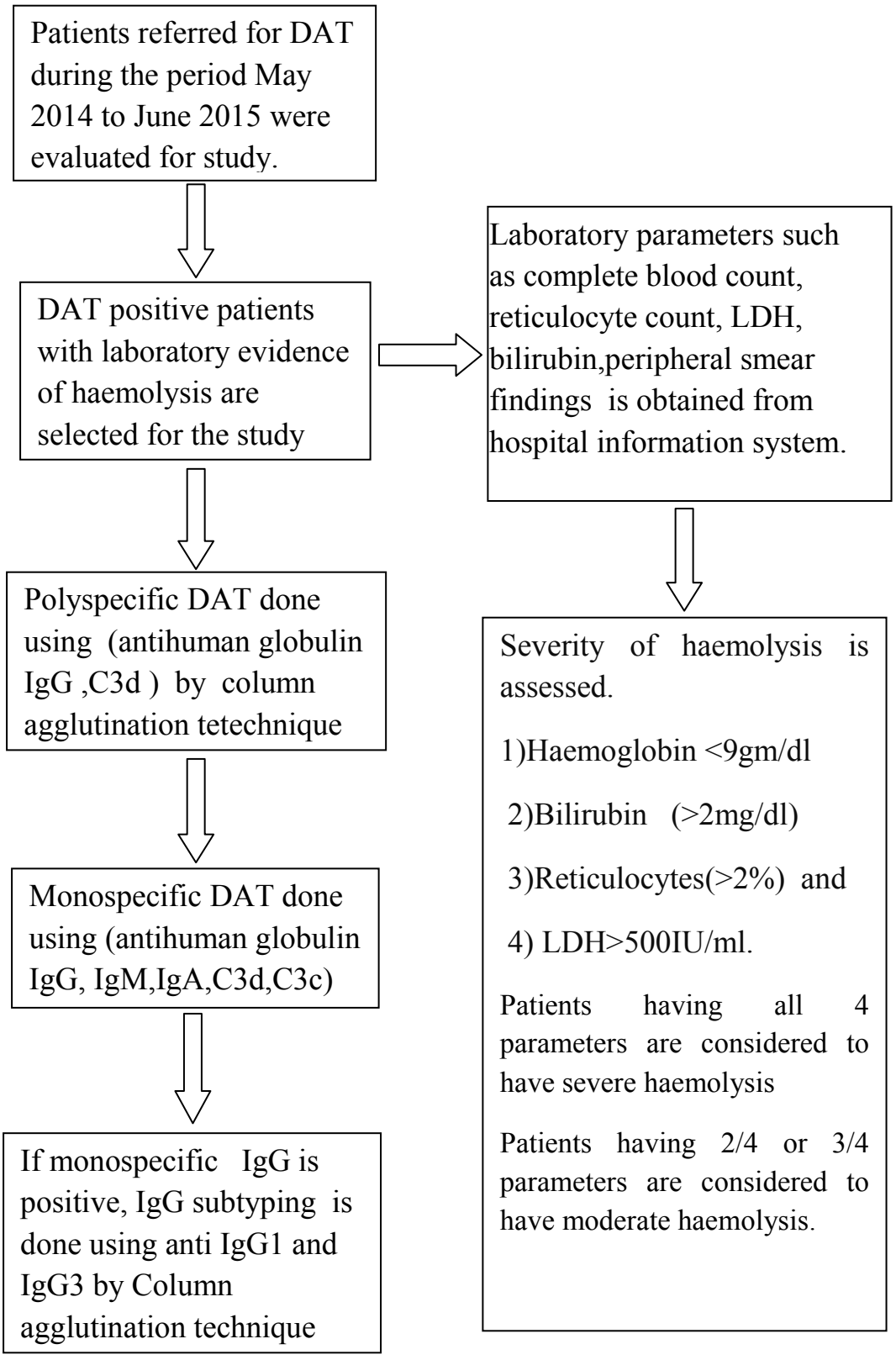
2) Neonates with Rh and ABO haemolytic disease

This study was carried out in the Department of Transfusion Medicine and Immunohaematology and the department of haematology. All DAT requests received in the blood bank between the period May 2014 and June 2015 were evaluated for study eligibility.All samples received in the blood bank for DAT are Ethylene diamine tetra acetic acid (EDTA) samples and were collected by evacuated blood collection tube.Polyspecific DAT was performed by CAT using LISS Coombs ID card "DAT IgG/C3d" which can detect IgG and C3d. Patients,who were positive for polyspecific DAT and had clinical and laboratory evidence of haemolysis were selected for the

study.The blood samples of patients' who could potentially be included in the study,was temporarily stored at room temperature and samples were processed within 24 hours of collection. Patients who had positive polyspecific DAT results were further evaluated by using monospecific DATby CAT using monospecific LISS Coombs ID card 'IgG,IgM,IgA,C3d,C3c (BIO-RAD)which could detect the presence of IgG, IgM, IgA, anti C3d and antiC3c. If monospecific IgG was positive, further IgGsub typingwas performed using antihuman globulin IgG1 and IgG3by CAT(from DiaMed GmbH 1785 Cressier FR Switzerland)(BIO-RAD) and this was done in two dilutions of 1:1 and 1:100.

Results of DAT were graded from 0 to 4 as permanufacturer's recommendations and strength of DAT was interpreted by two individuals (principal investigator and an experienced technologist) independently.A Clinical research form was filled using the patient's medical records and hospital information system. Patients were classified as having primary or secondary AIHA based on the history, laboratory and radiological results.Patients were classified to have severe or moderate haemolysis based on criteria laid down by Dass et al as described above.The study algorithm is shown below.

Figure1- Algorithm to demonstrate the methodology of this study



Principle of Direct Antiglobulin Test (DAT)

This DAT is based on the test developed by Coombs, Mourant, and Race for the detection of antibodiesattached to red cells that do not produce direct agglutination and these antibodies are called incomplete antibodies. Most of the antiglobulin reactivity is directed at the heavy chains (eg, Fc portion of the sensitizing antibody) or the complement component, thus bridging the gap between adjacent red cells to produce visible agglutination. The strength of the agglutination is usually proportional to the amount of bound protein. The DAT is performed by testing freshly collected red cells directly with antiglobulin reagents containing anti-IgG and anti-C3d.Eventhough any red cells can be tested, EDTA-anticoagulated blood samples are preferred,since EDTA prevents in-vitro fixation of complement by chelating the calcium that is needed for C1 activation. In this study DAT initially performed with a polyspecific antihuman globulin (AHG) reagent that is capable of detecting both IgG and C3d. If the results are positive, tests with monospecific reagents (anti-IgG,IgA,IgM and anticomplement) performed to appropriately characterize the immunoglobulins involved and determine the diagnosis.Monospecific IgG is positive subtyping of IgG was using IgG1 and IgG3 and the test done by column agglutination technique.

Column Agglutination technique for DAT: Lapierre et al, was the first to describe this technology which aimed to standardise red blood cell agglutination reactions and help in uniform interpretation of results. In this method red cells agglutinated are trapped at the top, which can be read and graded by naked eye.

One column usually consists of six special microtubes containing gel matrix or glass beads. Red cells alone for DATtesting or serum for ICT are added to microtubes. Depending on the test that needs to be carried out, in a specific concentration as per manufacturer and test protocol, followed by incubation when necessary, and then centrifuged under controlled parameters. The gel contained in each of the microtubes acts as a sieve, to trap the antigen antibody complexes formed, while the unagglutinated red blood cells passes through the gel and form a pellet at the bottom of the tube. The gel in the microtubes may be neutral or contain specific reagents like monoclonal antihuman globulin as in DAT test.(66)

It is presently commonly used platform in bloodbank, this test can be performed on this platform manually or by automated machine, which allow for processing multiple samples at the same time with decreased turnaround time. CAT can be used to perform blood grouping, antibody screening, DAT, Indirect antiglobulin test and cross-matching in blood banking.Antigen antibodyreaction occurs in the chambers at the top of a column, after incubation and centrifugation as recommended by the manufacturer, the test can be interpreted. Positive reaction is where the agglutinated red cells remain at the top, and negative reaction is where the free red cells are forced through the column to the bottom.

Standard operating procedure for polyspecific DAT by column agglutination technique (CAT).

Sample: Appropriately labelled 4ml EDTA sample.

Materials Required: Coombs card (Fig 2) ID Card Centrifuge Micropipette and tips,ID card holder, LISS,Heat block (Incubator)/ID card Incubator

Fig.2 Polyspecific DAT card (BIO-RAD)

Principle of Polyspecific DAT: To detect the presence of incomplete IgG and complement binding antibodies coated on patients' red cells. Cells coated with IgG and /or complementwill show agglutination with broad spectrum AHG reagent.

Procedure for polyspecific DAT using Bio-Rad card

1. Ensure that the cassette is at room temperature before use

2. Label the cassette appropriately with patient ID

3. Add 50µl of 0.8% suspension of patient cells suspended in LISSto polyspecific ID card.

4. Centrifuge the card in ID card centrifuge for 10 minutes at 910rpm

5. Read and record the reactions (read the card from both sides)

50 ul 0.8% of patient cells, prepared by suspending 10ul of packed cells in 1000ul of LISS (as per manufacturers recommendation),This cell suspension was added to polyspecific LISS Coombs DAT ID card which are impregnated with anti IgG, antiC3d .After addition of cells, cards are centrifuged for 10 minutes at 910rpm.

If patients were polyspecific DAT positive and also noted to have clinical and laboratory features suggestive of haemolysis were included in the study, monospecific DAT was performed as below.

Standard operatingprocedure for monospecific DAT.

Principle of monospecific DAT- To detect the presence of incomplete antibodies and complement binding antibodies coated on patients red cells which are IgG, IgM, IgA, c3d, and C3c using anti IgG, anti IgM, anti IgA, anti C3d and anti C3c respectively.

Material Required.ID-card with 6 columns (Fig 3 having 5 microtubes impregnated with anti IgG, anti IgM, anti IgA, antic3d,antic3c and a negative control microtube), Pipettes, Tips, Incubator and Centrifuge.

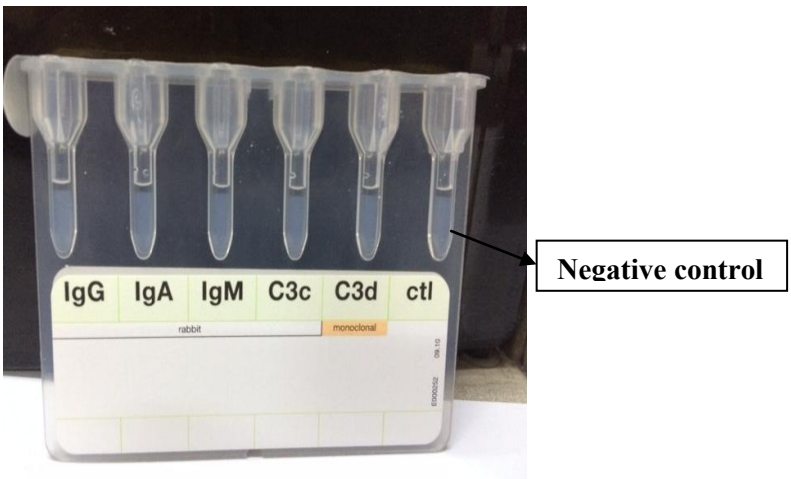


Fig.3Monospecific DAT card (BIO-RAD)

Procedure for monospecific DAT using Bio-Rad card

1. Ensure that the cassette is at room temperature before use

2. Label the cassette appropriately with patient ID

3. Add 50µl of 0.8% suspension of patient cells suspended in LISS to monospecific ID card.

4. Centrifuge the card in ID card centrifuge for 10 minutes at 910rpm

5. Read and record the reactions (read the card from both sides)

50 ul 0.8% of patient cells, prepared by suspending 10ul of packed cells in 1000ul of LISS (as per manufacturers recommendation),This cell suspension was added to monospecific LISS Coombs ID card which are impregnated with anti IgG, anti IgM, anti IgA, anti C3d and anti C3c respectively. After addition of cells, cards are centrifuged for 10 minutes at 910rpm.

If patients were noted to have monospecific IgG on monospecific DAT, further subtyping of IgG was done as below.

IgG1 and IgG3 subtyping:

Principle: The risk of haemolysis depends on the amount of IgG coated on the red cells as well as on the IgG subclasses involved. To induce phagocytosis it is suggested that approximately 1000-4000 IgG1 molecules or 135-500 IgG3 molecules must coat the red cells. Therefore it is not only important to identify the presence of these subclasses but may require an estimation of the titre levels to comprehend its clinical significance.

The ID-Card "DAT IgG1/IgG3" has two dilutions (1:1 and 1:100) of the both subclasses and works on the principle of CAT.

Materials: ID-card with 6 columns (having four microtubes for IgG 1 and IgG3 in two dilutions each, a negative control well, and a positive control well), Pipettes, Tips, Incubator and Centrifuge.

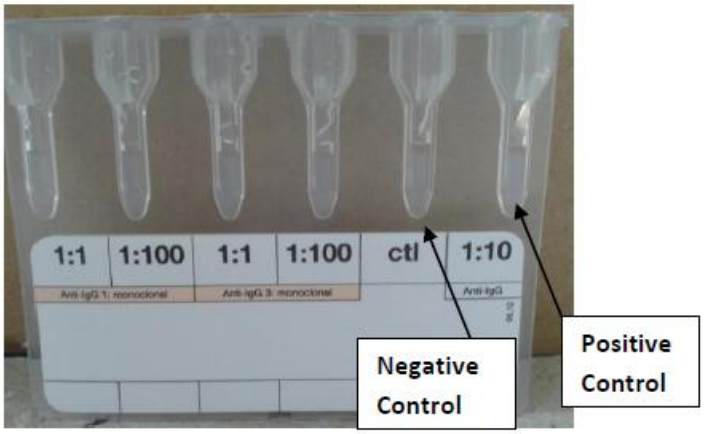


Fig.4 DAT IgG1/IgG3 card in 1;1 and 1:100 dilution

Procedure

1. Label the ID-card with appropriate patient hospital/identity number
2. Remove aluminium foil, holding the card in upright position
3. Pipette 50µl 0.8% LISS suspended patient cell into each micro tube.
4. Centrifuge the card for ten minutes.
5. Interpret and record the agglutination reaction

Interpretation:

A. Positive: Agglutinated cells form a red line on the surface of the gel (strong reaction) or the agglutinates tend to disperse in the gel in a weaker reaction.

B.Negative: Compact button of cells at the bottom of the microtube.

A positive reaction with the 1:1 dilution has a sensitivity of approximately 1000 IgG1 molecules and 125 anti-IgG3 molecules respectively. A positive reaction in 1:100 dilutions is an indicative of a higher strength of antibodies in the plasma.

The results of the test are graded as shown in figure 1**.** The reaction on the card was read from both the sides and interpreted by two individuals (principleinvestigator and the experienced technologist) independently to avoid interpretation error.

Grading of DAT reaction-

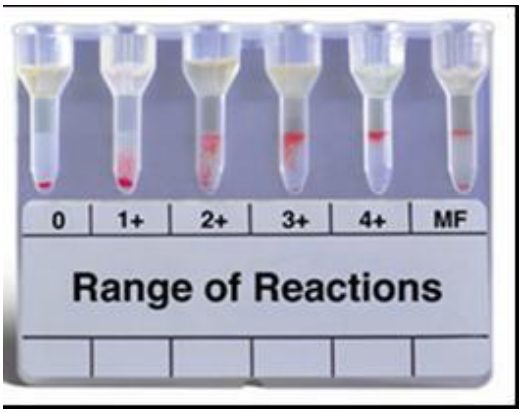


Fig-5 Grading of DAT reaction

1+ Reaction-Abutton of cells at the bottom with most aggregates remainingat the lower half of the column.

2+Reaction-Small button of cells at button of cells at bottom with aggregatesobserved throughout the length of the column

3+ Reaction- Most aggregates remains at the upper half of the column.

4+ Reaction--Agglutinated cells form a band at the top of the column

Weak Reaction- Granular suspension

MF Reaction - Mixed field agglutination / double population

Classification of severity of haemolysis.

Severity of haemolysis was classified into moderate or severe based on the following laboratory parameters.

1) Haemoglobin <9gm/dl

2) Bilirubin (>2mg/dl)

3) Reticulocytes (>2%) and

4) LDH>500IU/ml.

The haemolysis is classified into severe if all the above parameters were fulfilled, classified into moderate on the basis of whether two or three of the above laboratory parameters mentioned above are abnormal (3,55,65)

Clinical and laboratory data relevant to the study was collected from medical records and clinical workstation.These are age, gender, presence or absence of organomegaly, causes of secondary AIHA, first time diagnosis of AIHA or already diagnosed and on treatment or relapsed AIHA.

Bias:

All effortshave been made to eliminate any bias in selection of patients. Despite this, there may inherent risk of bias, considering CMC being a tertiary referral centre, thus attracting patients with complex clinical profile and greater severity of haemolysis.Instrument bias was likely to be negligible as variables were measured using calibrators and automated analyzers in the Clinical Pathology department and blood bank and all the serological tests done in the blood bank were run in duplicates. The column agglutination gel card, which was the novel test done in this study was interpreted by two individuals (principal investigator and an experienced technologist) independently. Investigator bias is avoided as the investigator is not involved in diagnosis /treatment.

SAMPLE SIZE:

Sample size estimated in the study is 56.

Sample size was calculated based on the only Indian study available byDas SS et al, which showed, 60% of the patients had severe haemolysis, whose red cells are coated with IgG1 or IgG3 or both as contrary to 21% ,when these IgG subclasses were absent.(55)

Two Proportion - Hypothesis Testing - Large Proportion - Unequal Allocation

Proportion in group I	0.6
Proportion in group II	0.2
Estimated risk difference	0.4
Power (1- beta) %	80
Alpha error (%)	5
Allocation Ratio	03:01
1 or 2 sided	2
Required sample size for group I	42
Required sample size for group II	14

Sample size was calculated using this formula: $n = \frac{\left(Z_{\alpha/2}+Z_{1-\beta}\right)^2 * 2 * PQ}{d^2}$

Where P = average of p1 and p2

Q = 100 – Pd = p1-p2

p1 = group1p2=group2

$Z_{\alpha/2}$=alpha error, $Z_{1-\beta}$ = power

However in our study we achieved a sample size of 94.

STATISTICAL ANALYSIS:

Median, mean, Standard deviation, and range was calculated for all continuous variables. Pearson and Fisher's exact chi square was used for comparison of categorical data. Mann-Whitney test is used to calculate p value of LDH, bilirubin and reticulocytes. Binary logistic regression analysis was used for comparing the categorical data and to calculatethe odds ratio with 95% confidence interval (CI). P value <0.05 was considered significant.

RESULTS

Total of 94 patients with clinical and laboratory features suggestive of AIHA were included in the study between the period May 2014 and July 2015.

On analyzing the time of presentation, patients enrolled belonged to different categories. These patients are

- First time diagnosed to have AIHA.
- Patients already diagnosed to have AIHA and on treatment at the time of analysis
- Previously diagnosed to have AIHA, currently relapsed after stopping treatment

All these category of patients were included in the study, since distribution of these cases were equal among the severity and type of AIHA (p=0.394)

Age distribution of patients

The age distribution of patients ranged from 1 year to 77 years with a median age of 35.2 years.

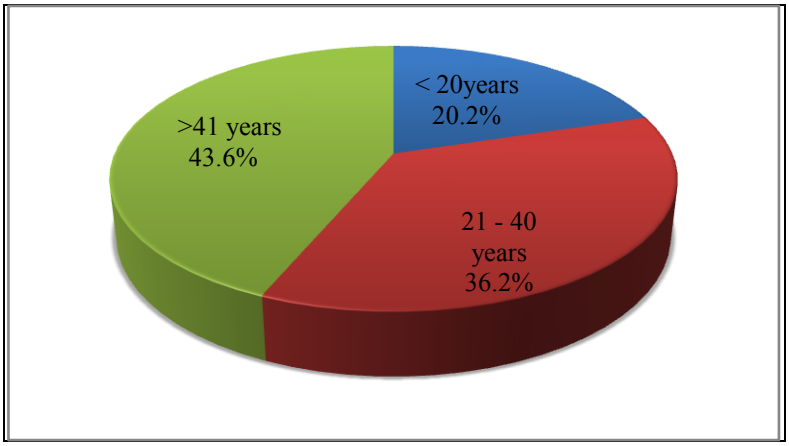


Fig. 6 Age distribution of patients

Age distribution of patients in primary and secondary AIHA

While comparing the age distribution of patients betweenprimary and secondary AIHA,median age was 45years (Range 1-77 years) in the former and26 years (Range of 2-70years) in the latter.Primary AIHA was predominantly noted in patients more than 40 years of age (52.9%), however secondary AIHA was frequently noted in patients less than 20 years (30%).

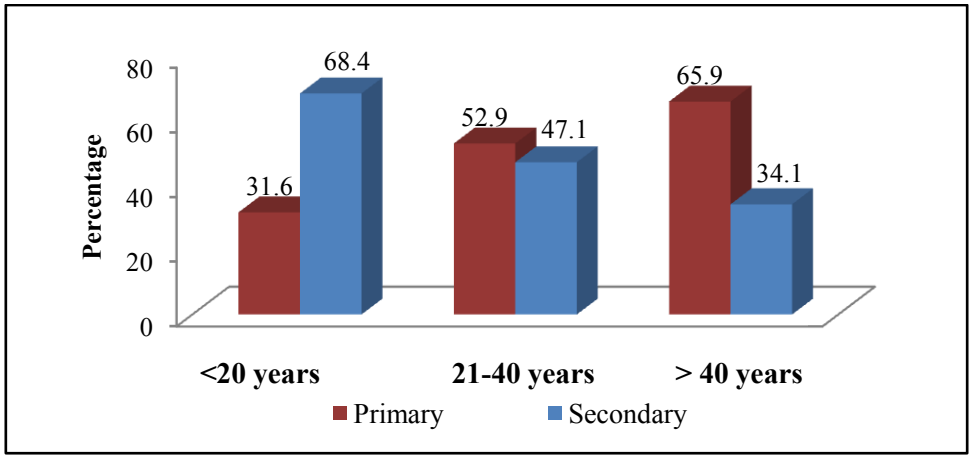


Fig. 7Age distribution of patients in primary and secondary AIHA

Gender distribution– In our cohort of 94 patients, 32(34%) patients were males and 62 (66%) patients were females with overall ratio of 1:1.9 (32/62) as shown in Fig 8.

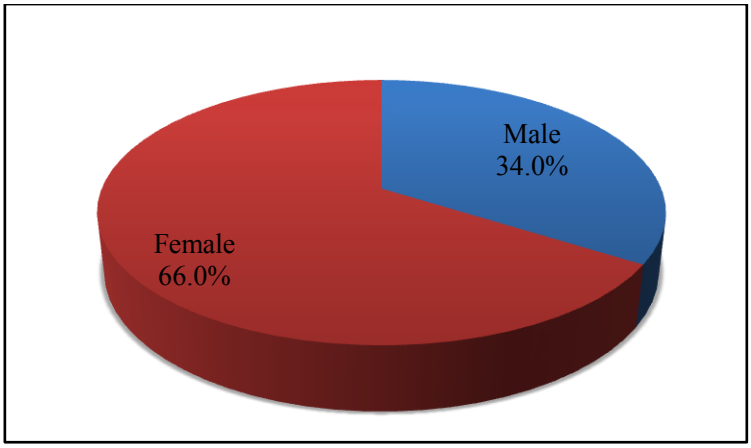


Fig8. Gender distribution in study population

Gender distribution in primary and secondary AIHA

Whilst comparing the gender distribution of patients between primary and secondary AIHA, Male: Female ratio was noted to be 1:1.4with primary AIHA and 1: 2.9 for secondary AIHA.

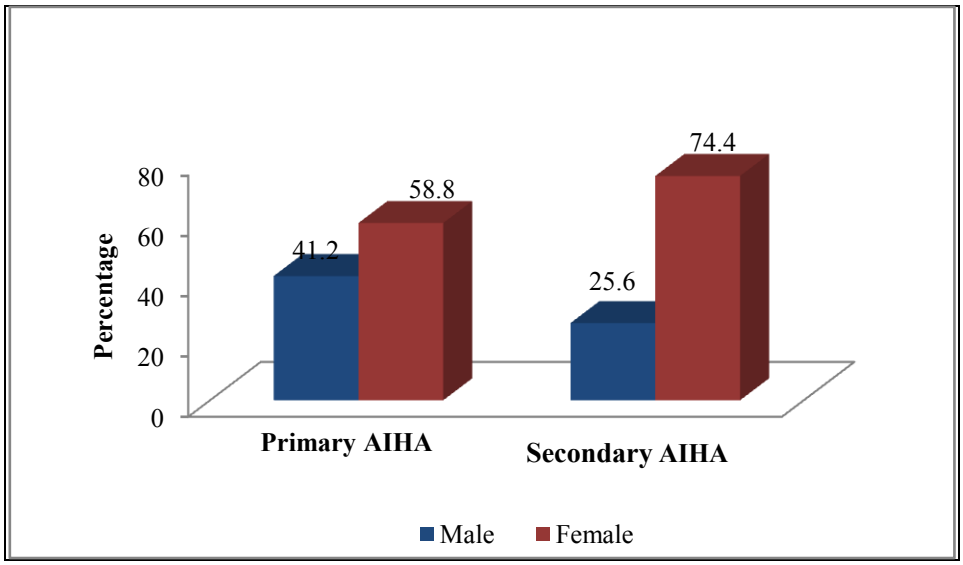


Fig 9 Gender distribution in primary and secondary AIHA

Type of AIHA- Outof 94 AIHA patients, 51(54.3%) patients were diagnosed to have primary AIHA and remaining 43(45.7%) patients had secondary AIHA.

The common causes of secondary AIHA noted are

1) Autoimmune disease
2) Lymphoproliferative disorder
3) Infection
4) Myeloma,
5) MDS and lymphoma
6) Vitamin B12 deficiency,
7) Homeopathy medicine intake.
8) Ethanol intake

Frequency of secondary causes of AIHA is shown in the table below.

Table2 .Various causes of secondary AIHA in study population

Causes of secondary AIHA	NUMBERS	PERCENTAGE
Autoimmune disease	28	65.1
Lymphoproliferative disorder	6	14.0
Infection	2	4.7
Other causes	7	16.3
Total	43	100.0

Other causes included areMyeloma, MDS and lymphoma, ethanol intake, infection vitamin B12 deficiency, homeopathy medicine intake.

Severity of AIHA.

Haemolysis was classified into severe and moderate based on study criteria laid down by Dass et al.Out of 94 patients, 52(55.3%) patients were categorised to have severe haemolysis and 42(44.7%) with moderate haemolysis category as shown below(fig11).

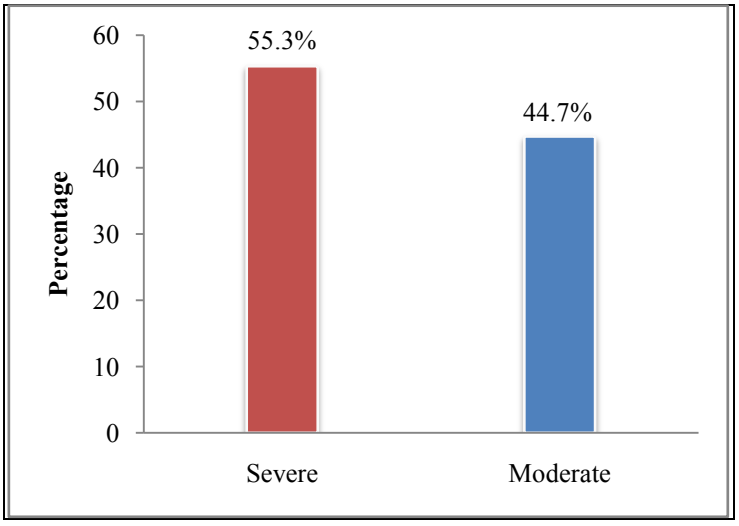


Fig 11.Percentage of patients with Severe and Moderate haemolysis.

Severity of haemolysis in primary and secondary AIHA patients.

Among 52 patients who had severe haemolysis, 71.2% (37) had primary AIHA and remaining 28.8% (15) had secondary AIHA. ***When we calculated association of primary AIHA with severe haemolysis using Fisher exact chi square test, it was statistically significant withp< 0.001(OR = 4.76, 95% CI 1.97-11.48).*** Among moderate haemolysis patients, majority of them had secondary AIHA as compared to primary AIHA with 66.7% (28) inthe former and 33,3%(16) in the latter.

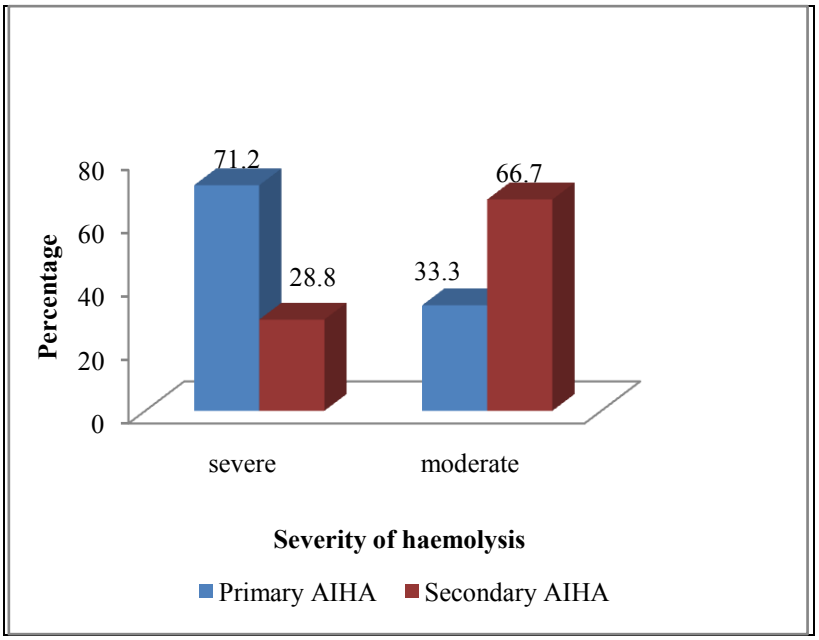


Fig12. Severity of haemolysis in primary and secondary AIHA

Mean haemoglobin value, in patients with severe and moderate haemolysis.

Mean value of haemoglobin was assessed in patients with severe and moderate haemolysis by T test. There was a statistically significant difference in mean haemoglobinin patients with severe and moderate haemolysis with haemoglobin of 5.9gm/dland 7.3gm/dl respectively.(*p value = 0.002)*

Median values Bilirubin, Reticulocytes and LDH in severe and moderate haemolysis.

Median values of Bilirubin, Reticulocyte and LDH were assessed in patients with severe and moderate haemolysis by Mann Whitney test. Differencein Median values of bilirubin, reticulocytes and LDH were noted to be statistically significant with p value <0.001.

Table3. Univariate analysisof Haemoglobin,Bilirubin, Reticulocytes and LDH in Patients' with severe and moderate haemolysis

Haemolysis	**Haemoglobin*** Mean (sd)gm/dl	**Bilirubin**** Median (range)mg/dl	**Reticulocyte**** Median(range)%	**LDH***Median (range)Iu/dl
Severe	5.9(1.8)	3.3(0.6-32.9)	13.85(2.6-45.2)	1153.(494-3634)
Moderate	7.3(2.2)	0.7(0.2-12.4)	3.00(0.1-21.5)	803.(273-3680)

Note: * p value = 0.002 --- T-test** p value = <0.001 – Mann-Whitney test

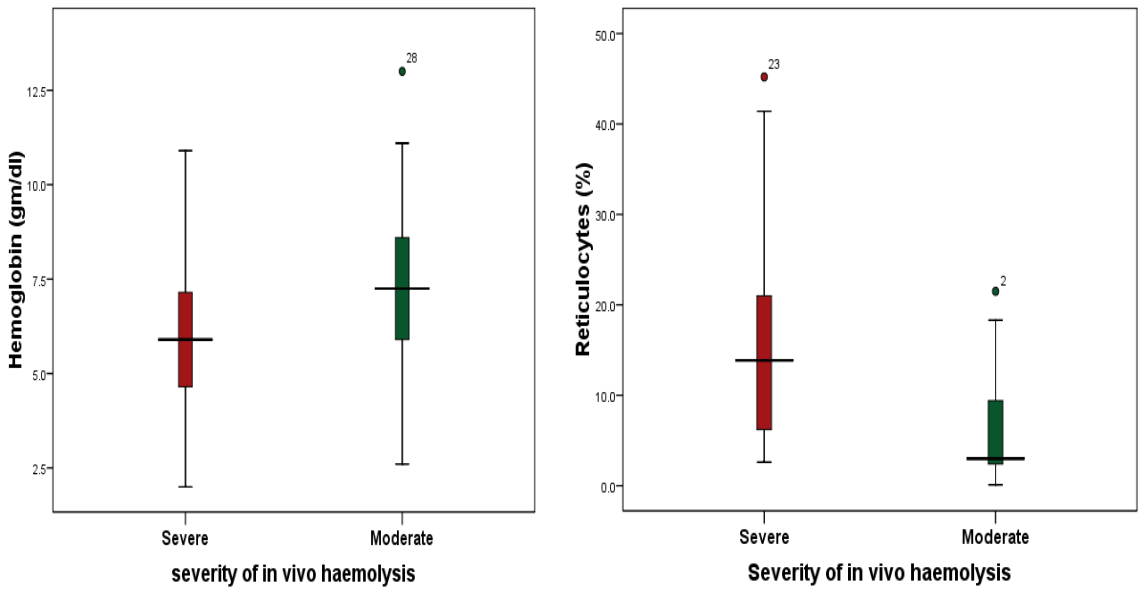


Fig13.Box plotsshowing haemoglobin and reticulocytes mean and median values respectively in patients with severe and moderatehaemolysis

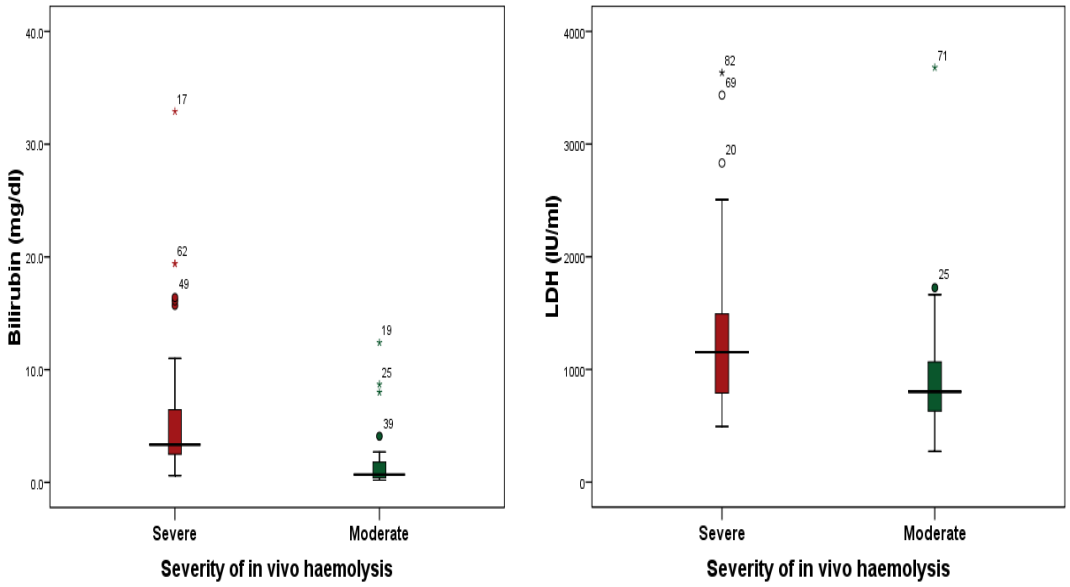


Fig14. Box plots showingLDH and bilirubin valuesrange and median values in patients with moderate and severe haemolysis.

Distribution of autoantibodies in patients

Among 94 patients, 62.8% (59) patients were identified to have multiple autoantibodies, ieIgG along with other autoantibodies or complement, as opposed to 28.7% patients who had solitary IgGalone. 8(8.5%) patients were identified to have complement alone, raising the possibility of cold agglutinin disease (as shown in the figure below).

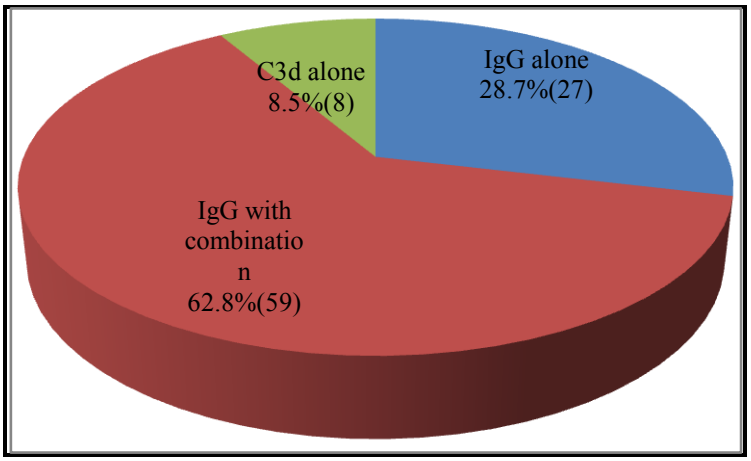


Fig15. Distribution of autoantibodies in patient population

Thevarious combinations of autoantibodies noted in our patients' population are shown below.

Table 4.Various combinations of autoantibodies in studypatients

Autoantibody type	Severe	Moderate	OR	95% CI	P value
	N= 52	**N= 42**			
IgG Only (n=27, 28.7%)	5(9.6)	22 (52.4)	0.097	0.032-0.291	<0.001
IgG + IgA (n=1, 1.1%)	1(1.9)	-			
IgG + IgM (n=1, 1.1%)	1(1.9)	-			
IgG + C3d (n=29, 30.9%)	20(38.5)	9(21.4)	2.292	0.909-5.778	0.079
IgG + IgM + C3d (n=15 , 16.0%)	10(19.2)	5(11.9)	1.762	0.552-5.626	0.339
IgG + IgA + C3d (n=2, 2.1%)	1(1.9)	1(2.4)			
IgG + IgM + C3c + C3d (n=8, 8.5%)	6(11.5)	2(4.8)			
IgG + IgA + IgM(n=1, 1.1%)	0	1(2.4)			
IgG + IgA + IgM+C3d (n=2, 2.1%)	2(3.8)	0			
C3d only (n=8, 8.5%)	6(11.5)	2(4.8)			

Impact of solitary IgG vs IgG in combination with other autoantibodies and/ or complement in patientswith haemolysis.

Of the 46 patients who had severe haemolysis,89.1%,(41) of patients had combination of autoantibodies, as compared to 10.9%(5) patients, who had solitary IgG. ***Logistic regression analysis showed that presence of multiple antibodies were more significantly associated with severe haemolysis (p<0.001).***

Table5. Distribution of solitary IgG vs IgG in combination with other autoantibodies in patients with haemolysis.

Autoantibody type	**IgG in combination**	**Isolated IgG**	**OR**	**95% CI**	**P value**
	N= 59	N=27			
Severe	41 (89.1%)	5 (10.9%)	10.022	3.276 – 30.6556	<0.001
Moderate	18 (45.0%)	22 (55.0%)	1.00		

Distribution of IgG subtypes.

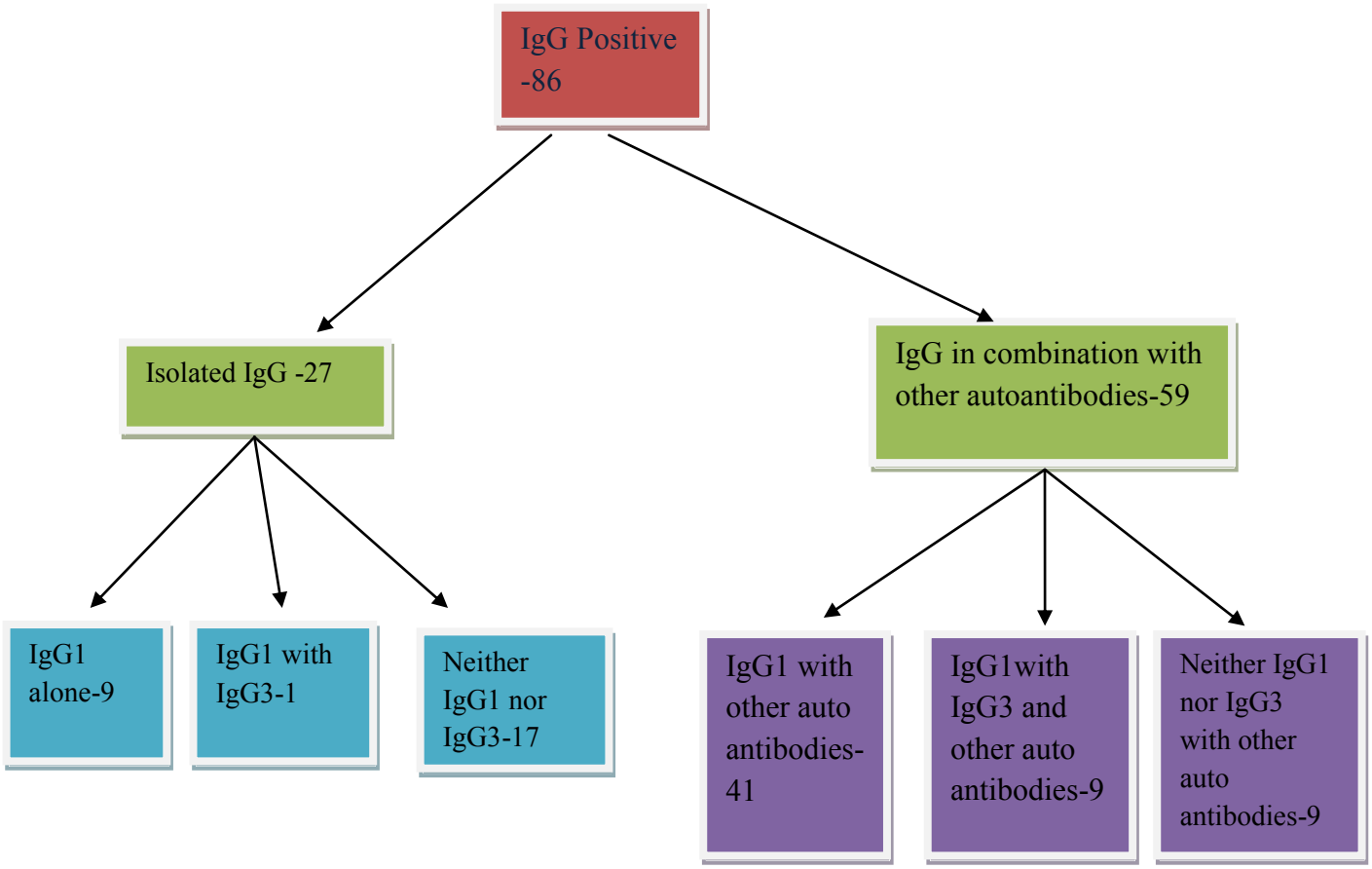


Fig 16 Flow Chart of distribution of IgG subtypes

Of the 86 patients who had IgG, 27 of them had solitary IgG as compared to 59 patients who had IgG in combination with other autoantibodies. (Detailed description on Fig 16).IgG1was the most common IgG subtype identified in 58.1% (50) of patients,as compared to11.6%(10) of patients,who were identified to have combination of IgG1 and IgG3. In this cohort of patients30.2% (26) did not show presence ofIgG1or IgG3 (Fig 17).

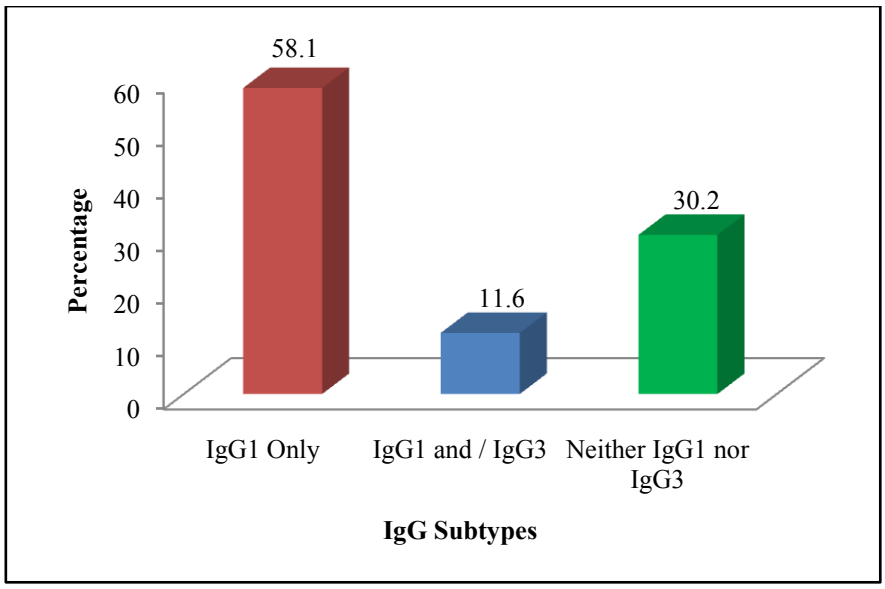


Fig 17.Distribution of IgG subtypes in patient population

Correlation of IgG subtypes with severity of haemolysis.

The presence of IgG1 and IgG3 in combination had significant impact on severity of haemolysis. A total of 10 patients were noted to have combination of IgG1 with IgG3.In this group 7(70%) patients, had severe haemolysis as compared to 3(30%) patients with moderate haemolysis. (p=0.04, OR 5.2 95% CI 1.34-10.08). A lesser but statistically significant impact was noted, when IgG1 was present alone. A total of 31(62%) patients had severe haemolysis as opposed to19 (38%) of patients with moderate haemolysis in patients with IgG1 alone. This association was statistically significant with p=0.012.

In patients who had neither IgG1 nor IgG3 subtypes, 69.2%(18) of patients had moderate haemolysis as compared to 30.8%(8) patients with severe haemolysis.

The logistic regression analysis of IgG subtypes with severity showed that patients with combination of IgG1 with IgG3 and IgG1 alone were 5.2 (95%CI- 1.0, 25.7) and 3.6(95% CI 1.3, 10.1) times respectively, more likely to present with severe haemolysis as compared to not having IgG1 and IgG3 respectively. (p=0.041, p=0.012 respectively) *as shown in the Table 10.*

Table 10 Correlation of IgG subclass with severity

IgG Subtypes	Moderate	Severe	Total	OR	95% CI	p Value
IgG1	19(47.5%)	31(67.4%)	50(58.1%)	3.671	1.34,10.08	0.012
IgG1 with IgG3	3(7.5%)	7(15.2%)	10(11.6%)	5.250	1.07,25.70	0.041
Absence of IgG1 and IgG3	18(45.0%)	8(17.4%)	26(30.2%)			
Total	40(100%)	46(100%)	86(100%)			

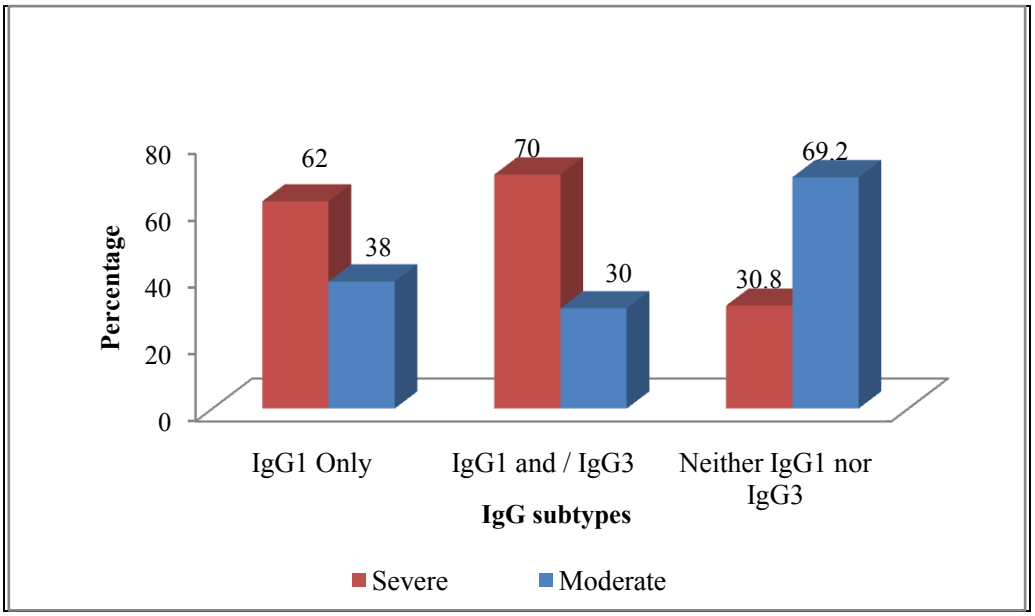


Fig 17 Correlation of IgG subtypes with severity

Clinical impact of IgG subtyping in patients with solitary IgG

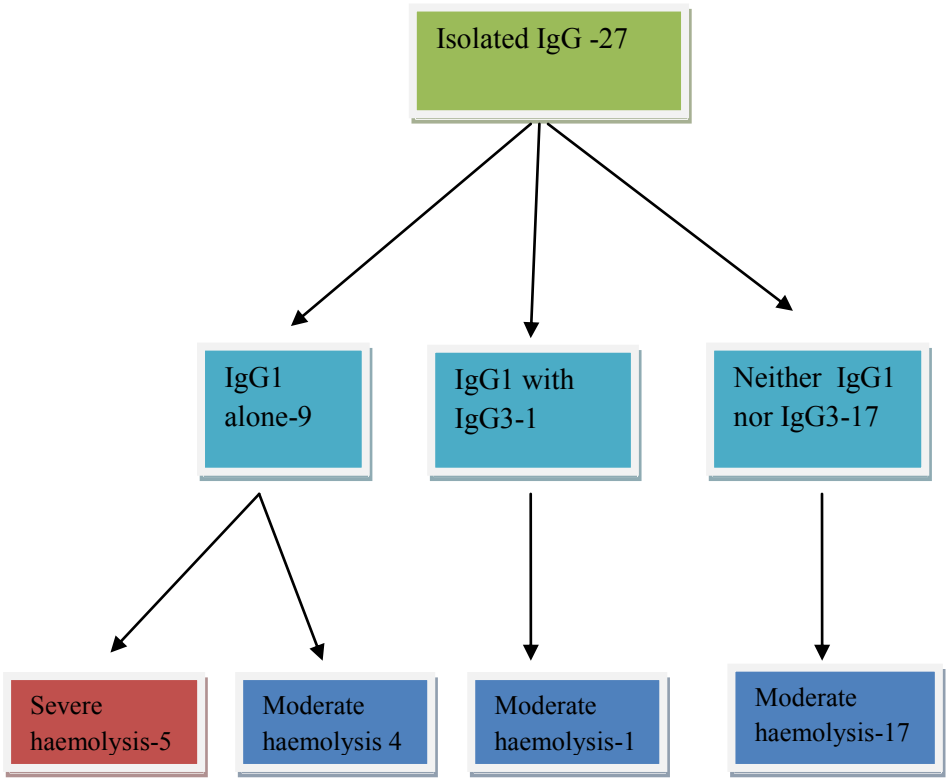


Fig18. Distribution of IgG subtypes in patients with solitary IgG

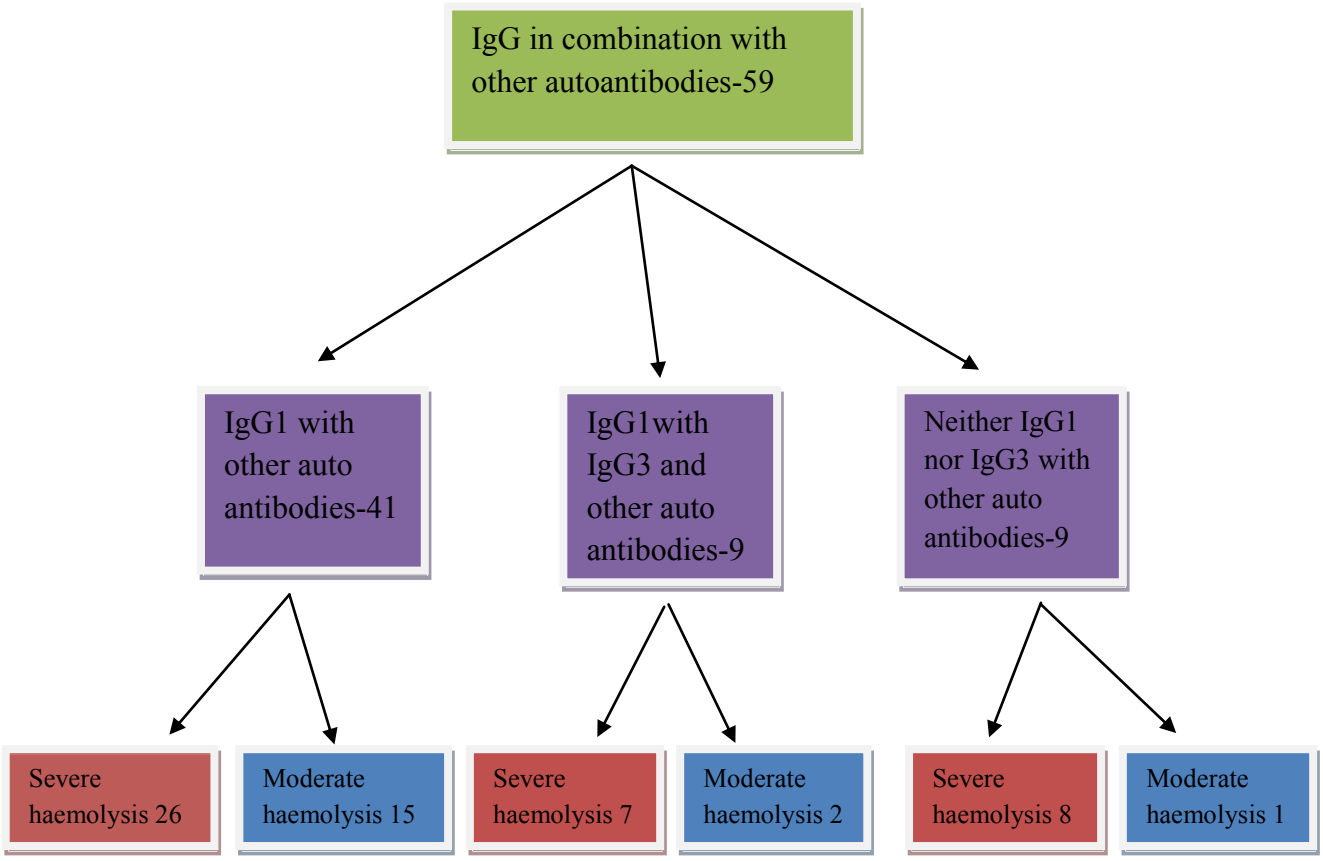
A total of 27 patients with solitary IgG were further subtyped and the distribution of further subtypes was as follows. 9 out of 27 patients were noted to have isolated IgG1of the 9 patients, 5 had severe haemolysis. Only one patient was identified to have IgG1in combination with IgG3 and had moderate haemolysis. There were no patients identified to have isolated IgG3.

A total of 17 patients, had neither IgG1nor IgG3 and all these patients had moderate haemolysis. This association of presence of IgG1and/ or IgG3 with patients who had neither IgG1 nor IgG3 was statistically significant with ap < 0.001(table8).

Table8. Impact of IgG subtypes in patients with solitary IgG

	Moderate AIHA	**Severe AIHA**	Total	P value
IgG1 alone	4 (44.4%)	5(55.6%)	9	P < 0.001
IgG1 with IgG3	1(100%)	0	1	
Absence of IgG1, IgG3	17(100%)	0	17	
Total	22(81.5%)	5(18.5%)	27	

Clinical impact of IgG subtypes in patients with combination of autoantibodies.



Flow chart19. Clinical impact of IgG subtypes in patients with combination of autoantibodies inclusive of complement

IgG in combination with other autoantibodies and /or complement was identified in 59 patients. On IgG subtyping three groups of patients were identified in this cohort of patients.

1. In this first category, 41 patients were noted to have IgG1 with combination of other autoantibodies and/or complement. Of the 41 patients 26 (63%) patients had severe haemolysis as compared to15 (36%) patients with moderate haemolysis.

2. This second group was contributed by combination of IgG1 and IgG3 in association with other autoantibodies and /or complement, was noted in 9 patients. Amongst them 7 (77.8%) had severe haemolysis as opposed to 2(22%) patients who had moderate haemolysis. Logistic analysis showed that patients with severe haemolysis were associated with the presence of combination of IgG1 with IgG3, however this was not statistically significant (***p value = 0.699***).

3. In third group,9 patients had neither IgG1 nor IgG3.Amongst them 8 (88.9%) patients had severe haemolysis and interestingly all these patients showed evidence of complement fixation, except one patient who was noted to have IgM along with IgG (shown in the flow chart 19).

The association of IgG subtypes in combination with other autoantibodies with severity of haemolysis was not statistically significant in this cohort of patients (p=0.203) (table6).

Table6. Clinical impact of IgG subtypes in patients with combination of autoantibody.

Subtype of IgG with other autoantibodies	Moderate haemolysis	Severe haemolysis	Total	P value
IgG1	15(36.6%)	26(63.4%)	41(100%)	0.203
IgG1 with IgG3	2(22.2%)	7(77.8%)	9(100%)	
Absence of IgG1 and IgG3	1(11.1%)	8(88.9%)	9(100%)	

Impact of IgG subtypes and complement fixation on severity of haemolysis.

This group of patients were noted to have different subtypes of IgG along with complement fixation. These patients were analysed to assess the impact of IgG subtypes on severity of haemolysis.A total of 56 patients were noted to have IgG autoantibodies and also fixed complement. Amongst them, few patients were identified to have IgM(26) and IgA(3) along with IgG and complement. On IgG subtyping IgG1 and /or IgG3 was identified in 48 patients and remaining 8 patients had neither IgG1nor IgG3. In the former group 32(66.6%) had severe haemolysis as opposed to 16(33.3%) patients with moderate haemolysis.In the latter group, 7 patients had severe haemolysis as compared to 1 patient with moderate haemolysis.This association was not statically significant with $p = 0.413$

Table 7.Impact of IgG subtypes along with complement on severity

	Moderate haemolysis	**Severe haemolysis**	**Total**	**P value**
Patients with IgG1 and/or IgG3 and complement	16(33.3%)	32(66.6%)	48	=0.413
Patients with neither IgG1 nor IgG3 and complement	1	7	8	

Impact of complement fixation in patients with IgG1 and/or IgG3 subtypes.

We studied the clinical impact of complement fixation in patients, with IgG1 and /or IgG3 subtypes.10 patients were noted to have IgG1 and /or IgG3 subtypes, without complement fixation as compared to 49 patients who had complement fixation.(Table9).This association was not statistically significant (p=0.306)

Table9. **Impact of complement fixation in patients withIgG1 and/or IgG3 subtype**

	Moderate haemolysis	**Severe haemolysis**	**Total**	**P value**
Patients with IgG1 and /or IgG3	5	5	10	0.306
IgG1 and /or IgG3 with complement	16	33	49	

Impact of complement fixation in patients who had neither IgG1 nor IgG3 subtypes.

We analysed group of patients, who were positive for IgG and negative for IgG1 and IgG3 subtypes. In this group we studied the impact of complement fixation and absence of it.

A total of 8 patients showed complement fixation as compared to 17 patients who did not show complement fixation. In the former group, 7 patients had severe haemolysis as opposed to one patient with moderate haemolysis. In the latter group, all 17 patients were noted to have moderate haemolysis.This association was statistically significant with a $p<0.001$. (table7)

Table7. Clinical impact of complement fixation in patients with IgGwith neither IgG1 nor IgG3

	Moderate haemolysis	**Severe haemolysis**	**Total**	**P value**
Patients with neither IgG1 nor IgG3	17	0	17	<0.001
Patients who fixed complement with neither IgG1 nor IgG3	1	7	8	

Strength of Polyspecific DAT with Severity

Correlating strength of DAT with severity of haemolysis revealed that, 80.8% of patients who had severe haemolysis also had a DAT strength of 4+ as compared to 52.4% of patients who had moderate haemolysis(p =0.006). 47.6% (20) of patients who had DAT strength of 3+ and 2 + had moderate haemolysis as compared to 31.2% (10) patients with severe haemolysis (fig 18).

Logistic regression analysis revealed, patients with DAT strength of 4+ were 9.5 (95% CI 1.921 – 47.441) times more likely to have severe haemolysis, compared to patients who had 2+ DAT reactions. This correlation was statistically significant with a P value of 0.006

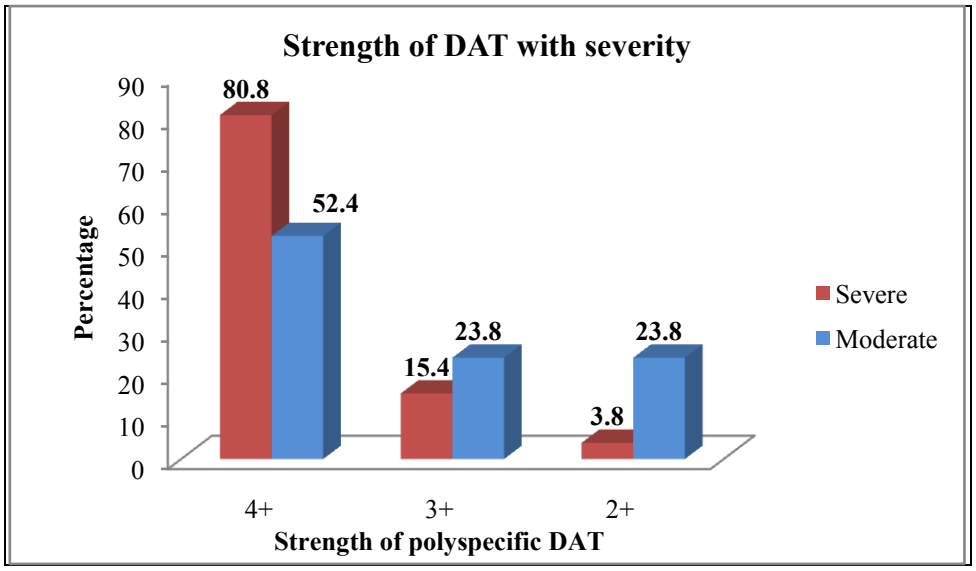


Fig18 .Correlation of the strength of DAT with severity of haemolysis

Table14.The strength of DAT with severity of haemolysis

Polyspecific DAT		Severe	Moderate	OR	95% CI	p value
	N=94	N= 52	N= 42			
4+	64(68.1)	42(80.8)	22(52.4)	9.545	1.921 – 47.441	0.006
3+	18(19.1)	8(15.4)	10(23.8)	4.000	0.674 – 23.725	0.127
2+	12(12.8)	2(3.8)	10(23.8)			

Correlation of strength of DAT with severity of haemolysis in patients with absence of IgG1 and IgG3

In patients with absence of IgG1 and/or IgG3, correlation of strength of DAT with severity of haemolysis revealed that,66.7% of patients with severe haemolysis had DAT strength of 4+ as compared to 33% of patients with moderate haemolysis. In patients who had moderate haemolysis, 80% of them had DAT strength of 2+ or 3+. This correlation was statistically significant with p=0.02

Table 15. Correlation of strength of DAT with severity of haemolysis in patients with absence of IgG1 and IgG3.

Strength of DAT	Moderate haemolysis	Severe haemolysis	P value
4+	2	4	0.02
3+	7	4	
2+	9	0	

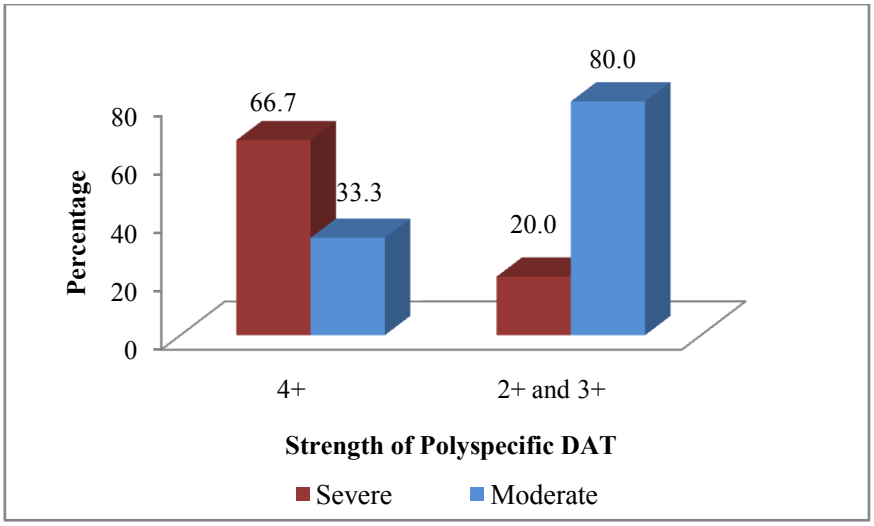


Fig 13.Strength of DAT with severity of haemolysis in patients with neither IgG1 nor IgG3

Impact of DAT strength on severity of haemolysis in patients with IgG1 and/or IgG3 subtypes.

50 patients who were positive for IgG1 and/ or IgG3 were analysed, taking into considerationof the following parameter.

- Presence of a particular subtype of IgG
- Dilution factor
- DAT strength in each of this subgroup.

Correlations were drawn, to assess DAT strength in association with subgroup with severity of haemolysis. Patients who had DAT strength of 3+ and 4+ in 1:1 IgG1 dilutions, were 2 times more likely to have severe haemolysis as compared to patients with moderate haemolysis. . However this was not statistically significant (p=0.330). Patients who had DAT strength of 3+, 4+ reaction with IgG1 subtype in 1:100 dilution were 4 times more likely to have severe haemolysis as compared to patients with moderate haemolysis. (Table 13 for more detailed description).In patients with IgG1

with IgG3 combination association of DAT strength with severity could not be analysed because of the smaller number

Table13: Impact of DAT strength on severity of haemolysis in patients with IgG1 and/or IgG3 subtypes.

Autoantibody type	**Severe** N= 31	**Moderate** N= 19	OR	95% CI	P value
IgG present (n=86)					
IGG1 only (n=50)					
Strength of DAT 1:1					
Present (1 and 2)	6(50.0)	6(50.0)			
Present (3 and 4)	25(65.8)	13(34.2)	1.923	0.516-7.164	0.330
Strength of DAT 1:100					
Absent (0)	14(50.0)	14(50.0)			
Present (1 and 2)	5(71.4)	2(28.6)	2.500	0.413-15.115	0.318
Present (3 and 4)	12(80.0)	3(20.0)	4.000	0.923-17.329	0.064
IGG1 + IGG3 (combination)(n=10)					
IGG1					
Strength of DAT 1:1					
Present (3 and 4)	7(70.0)	3(30.0)			
Strength of DAT 1:100					
Absent (0)	1(33.3)	2(66.7)			
Present (1 and 2)	2(66.7)	1(33.3)			
Present (3 and 4)	4(100)	-			
IGG3					
Strength of DAT 1:1					
Present (1 and 2)	-	1(100)			
Present (3 and 4)	7(77.8)	2(22.2)			
Strength of DAT 1:100					
Absent (0)	2(66.7)	1(33.3)			
Present (1 and 2)	3(100)	-			
Present (3 and 4)	2(50.0)	2(50.0)			

DISCUSSION

Autoimmune haemolytic anaemia(AIHA)is a collective term for several diseases characterized by autoantibody-initiated destruction of red blood cells (67).It is a relatively rare disease with varying clinical presentation. It is greatly heterogeneous, with symptoms ranging from fully compensated to patients presenting with fulminant, rapid onset of life-threatening anaemia(67).Immunoglobulin class, subclass, titre, ability to activate complement, thermal amplitude and strength of direct antiglobulin Test (DAT) have been implicated as factors affecting the severity of the disease(38,65). In view of this, it becomes very important to identify patients who are at risk of severe haemolysis, so that treatment and management is initiated under close supervision. With this background this study was undertaken, to serologically characterize the auto antibodies resulting in AIHA in the Indian population and to analyse the correlation between severity and the various factors implicated in AIHA.

AIHA is a rare disease.Based on population studies, the incidence of AIHA is 0.8 to 1/ 80,000 to100 000/year in western population(6,7) and the reported prevalence is 17/100 000(68) In literature there are no population based studies for incidence and prevalence of AIHA available from India. In our study prevalence of AIHA could not be assessed, since this was not a population based study and additionally, CMC is a tertiary referral centre for haematological disorders for many parts of the country.

It is known from various studies that frequency of AIHA is usually greater in females than in males (6). Similar trend was seen in our study population, with a male: female ratio of 1:1.9. Of the total 94 patients, 51(54.3%) patients were diagnosed to have primary AIHA and the remaining 43(45.7%) patients were noted to have secondary AIHA.This finding is in accordance with an Indian study by Naithani et al where the authorsnoted frequency of primary AIHA to be 65% and remaining 35% were secondary AIHA(69).Primary AIHA affects all age groups with the peak incidence of disease in the fourth and fifth decades. Secondary AIHA reflects the age distribution of the underlying disease. For example, inthe case of SLE, AIHA tends to present in much earlier age group and if AIHA is secondary to lymphoproliferative disorder tends to present in a relatively older age group.

In our study, the median age group of primary AIHA patient was 45years (Range 1-77 years), and that of secondary AIHA was noted to be 26 years (Range of 2-70 years).The most common secondary cause of AIHA was autoimmune disorders (SLE) at 68%, which was reflected by the younger age group of distribution of patients in secondary AIHA, followed by lymphoproliferative disorders (14%).

Haemolysis was classified into severe and moderate based on study criteria laid down by Dass et al(55). Of the total 94 patients, 52(55.3%) patients were categorised to have severe haemolysis as opposed to 42(44.7%) with moderate haemolysis.Severe haemolysis was greater in patients with primary AIHA (71.2%) as compared to patients with secondary AIHA (28.7%) The association of primary AIHA with severe haemolysis was statistically significant with $p<0.001$.

Majority of our patients had multiple autoantibodies (62.8%), ie IgG along with other autoantibodies and/or complement, compared to patients who had solitary IgG (28.7%)only. This is in contrast to the finding noted in Dass et al study, where 68.5% of their patients had solitary IgG(55).Study by Lai et al on AIHA patients, noted 44.4% with solitary IgG as opposed to 55.6% of patients with multiple autoantibodies. In our study we had 8(8.5%) patientswith complement alone, raising the possibility of cold agglutinin disease.

We also noted that,89.1% of our patients who had combination of autoantibodies had severe haemolysis as compared to 10.9%(5) patients, who had solitary IgG.This association was statistically significant ($p<0.001$).This finding is in accordance to the study done by Wheeler et al, and Dass et al respectively(38,55).

Patients with IgG, subsequently had subtyping using IgG1 and IgG3 card by column agglutination technique.This allowed identification of IgG1 and IgG3 in 1:1 and 1:100 dilutions. In our study, IgG1 was the most common IgG subtype, identified in 58.1% (50) of patients, as compared to 11.6% (10) of patients, who were identified to have combination of IgG1 and IgG3. In this cohort of patients 30.2% (26) did not show presence of IgG1or IgG3.

Study by Garrathy et al noted that, IgG1 found in the majority of patients with AIHA, IgG3 alone has been found in only 3% of patients and in combination with other subclasses in 5%.(46). Study done by Lai et al showed among 52 patients , IgG1 was detected in 53.8%, IgG3 in combination in 34.6%, and IgG3 alone in 7.7%, the IgG subclasses were negative in 3.8%.(70)As opposed to the finding in Lai et al study, we

did not haveisolated IgG3 and this subtype IgG3 was always found in association with IgG1. Interestingly in our study group we had 30.2% of patients with neither IgG1 nor IgG3 in comparison to Dass et al and Lai et al study where they had 51.2 % and 3.8% respectively (55,70)

When the correlations was drawn between the subtypes of IgG and severity of haemolysis.It revealed that patients with combination of IgG1 with IgG3 were 5.2 (95%CI- 1.0, 25.7) times more likely to present with severe haemolysis as compared to patients not having either IgG1 or IgG3 (p=0.041).In patients with IgG1 subtype alone, the odds of developing severe haemolysis was 3.6 (95% CI 1.3, 10.1) times more likely as compared to patients not having either IgG1 or IgG3 (p=0.012).

Similar findings were noted in the study done by Dass et al, where 60% of patients had severe haemolysis when their red cells are coated with IgG1 and / or IgG3 compared to 21% of patients who didn't have IgG1 or IgG3.Study done by Lai et al demonstrated that IgG3 autoantibody was the most effective antibody for red cell destruction followed by IgG1 autoantibody.IgG2 was noted to be even less potent and IgG4 was shown to hardly affect red cell survival(71).Study from Zhang et al on 84 AIHA patients, noted that IgG1 was the basic predominant IgG subclass detected in this group of patients and IgG3 had a greater potential for red-cell destruction than IgG1 and C3d(72).

Clinical impact of complement fixation.

We analysed the clinical impact of complement fixation on severity of haemolysis in three categories of patients. These are as follows

- Category 1 included patients who are positive for IgG and also had complement fixation.Patients with IgG were furthersubtyped .Logistic regression analysis was done to compare the group of patients with IgG1 and/or IgG3 and with patients with absence IgG1and IgG3. Interestingly this was not statistically significant (p=0.413)**,** likely explanation being *once complement fixation has occurred, the risk of severity increases irrespective of the IgG subtypes.*
- Our second category included patients withIgG1and/or IgG3 subtypes with or without complement.Analysis of this group revealed that association of IgG1 and /or IgG3 with or without complement fixation was not statistically significant (p=0.306) indicating that***, complement fixation did not impact significantly on severity of haemolysis in this group of patients.***
- Thethird category of patients included those who were positive for IgG and negative for IgG1 and IgG3 subtypes.Westudied the impact of complement fixation and absence of it, in this group of patients. ***Analysisof this group revealed that presence of complement fixation impacted significantly on severity of haemolyis (*p<0.001).**

The impact of complementon severity of haemolysis, as observed in our study isin accordance with previous literature published. A study by Wheeler et al on patients with AIHA documented that 72% of patients who had haemolysis also fixed

complement(3).Similar finding by Lin JS et al on 57 patients, in this study autoantiodies characterization was done using flowcytometric DAT, which showed synergistic effect of the red blood cell-bound IgG and complement in predicting hemolysis(73).A Study done by Dass et al documented that in 76.5% of patients, whose RBCs were coated with more than one type of immunoglobulin and complement had hemolysis.(49)

Correlation of strength of Polyspecific DAT with severity of haemolysis.

The strength of DAT positivity as expected correlates with number of immunoglobulin molecules adherent to the red cell. Literature mainly deals with the number of molecules of specific IgG1 subtypes that reflects as DAT positivity with or without haemolysis. A

Study done by Stratton et al showed that subjects whose red cells are coated with less than 950 IgG1 molecules per cell showed a positive DAT but no signs of red cell destruction. On the other hand, patients who had more than 1200 IgG1 molecules per cell were found to have significant haemolysis(51).This implies strength of DAT could correlate with severity of haemolysis.The fact that has been observed in the previous described studies.

To assess the strength of DAT,various platforms have been used these are tube method gel method ,flow cytometric DATetc.In literature many studies have been done to assess the correlation of strength of DAT with severity of hemolysis by various methods. In our study, polyspecific DAT, monospecific DAT and IgG subtype was performed by CAT method ,which was introduced by lapierre et al in the year

1990(74).CAT seems to be a robust and sensitive platform as described by Dittamar et al. In this study,they compared the efficacy of detection of DAT positivity on CAT vs. flow-cytometry, and showed that CAT was an equally sensitive platform to detect red cell bound antibodies(1).

A study done by Sudipta et al comparing CAT and 'tube technique' in AIHA patients demonstrated that CAT offers better sensitivity than other platforms which are used in immunohaematology(2).Study done by Dass et al using gel technique on DAT positive patients, showed gel test can be performed effectively in blood centers as a replacement to the conventional tube technique.(49)

There are many western studies which have studied the correlation between strength of DAT and *invivo* haemolysis. However there are very few studies from India regarding this and some of them have published contradicting results.Currently the strength of DAT is not used for assessing the severity of haemolysis.

In our study DAT strength of 4+ was strongly associated with severe haemolysis. Majority of our patients (80.8%) who had severe haemolysis also had a DAT strength of 4+ as compared to 52.4% patients with moderate haemolysis. Patients with DAT strength 4+ were 9.5 (95%CI 1.921 – 47.441) times more likely to have severe haemolysis, compared to patients who had 2+ DAT strength of reaction(p=0.006).Additionally patients with DAT strength of 3+ were 4 times (95% CI 0.674 – 23.725) more likely to have severe disease, although this was not statistically significant (p =0.127)

In a study published by Wheeler et al(3), DAT strength of 2 + or more strongly correlated with haemolysis and a similar study from Wikman et al also showed a

strong correlation between the strength of DAT and severe haemolysis(4). Studydone in AIIMS by Choudhary et al correlating the strength of DAT with severity of haemolysis show contradicting results. They did not find any correlation between DAT positivity and severity of anaemia(4). Study done by Dass et al in the year 2008 demonstrated that greater strength of DAT was associated with increased severity of haemolysis(55).

As a subgroup we also analyzed the correlation of strength of DAT with severity of haemolysis in patients with absence of IgG1 and IgG3 subtypes. Majority of patients (66.7%) with severe haemolysis had DAT strength of 4+ and this association was statistically significant (p=0.02). This clearly reveals that the DAT can be used as a tool to assess severity of haemolysis in AIHA patients irrespective of the IgG subtypes.

Analysing strength of DAT on severity of haemolysis in patients with IgG1 and/or IgG3 subtypes in 1:1 and 1:100 dilution revealed increase odds of developing severe haemolysis by 2 times and 4 times compared to patients with moderate haemolysis respectively. However this was not statistically significant. Study by Lai et al did not find correlation between the strength DAT and haemolysis in patients with IgG1 and IgG3 subtypes.

In view of the heterogenous manifestion of AIHA, identifying patients at risk of severe haemolysis is critical for prognostication, appropriate intervention and follow up planning. In our study, primary AIHA, presence of multiple autoantibodies, presence of IgG1 and or IgG3 subtypes impacted on severity of haemolysis and was

found to be statistically significant. In addition the presence of complement fixation in patients negative for IgG1 and IgG3 and DAT strength of 4+ correlated significantly with severity of haemolysis.

Hence we suggest, in patients with AIHA an algorithm of following up DAT positivity, with monospecific DAT, defining strength of reactivity of DAT and analysing IgG subtypes, will allow for identification of this critical subgroup of patients who are more prone to severe haemolysis, in whom more intense clinical intervention and close follow up might be indicated.

LIMITATIONS OF THE STUDY

1. This study does not represent the true prevalence of AIHA, since CMCis a referral centre.

2. Criteria for assessing severity of haemolysis was derived from a sigle previous publication from Dass et al, as no clinical guidelines available for the same.

3. Patient recruited for the study belonged to different time points in the course of their disease.

CONCLUSION

1. The profile of autoantibodies identified in patients with AIHA , in our cohort of patients was predominantly IgG along with complement followed by a few cases of complement alone and occasional cases with IgM and IgA

2. The parameters, which had the greatest impact on severity of haemolysis included are, diagnosis of primary AIHA,presence of multiple autoantibodies, presence of IgG1 and IgG3 subtypes,complement fixation and strength of DAT.

3. The presence of IgG1 and IgG3 subtypes assumed statistical significance when detected in the absence of complement. Those with IgG1 and IgG3 subtypes had significantly greater risk of severe haemolysis when compared with patients having negative for the same.

4. This association in our study of DAT strength, IgG1 and IgG3 positivity, and complement fixation on severity of haemolysis suggest that an algorithm of following up DAT positivity, in patients with AIHA, with a monospecific DAT and IgG subtype analysis will allow for identification of this critical subgroup of patients in whom more intense clinical intervention and close follow up might be indicated.

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ANNEXURE

OFFICE OF RESEARCH
INSTITUTIONAL REVIEW BOARD (IRB)
CHRISTIAN MEDICAL COLLEGE, VELLORE, INDIA.

Dr. B.J. Prashantham, M.A., M.A., Dr. Min (Clinical)
Director, Christian Counseling Center,
Chairperson, Ethics Committee.

Dr. Alfred Job Daniel, D Ortho, MS Ortho, DNB Ortho
Chairperson, Research Committee & Principal

Dr. Nihal Thomas,
MD., MNAMS., DNB (Endo), FRACP (Endo), FRCP (Glas) (EDIN)
Deputy Chairperson
Secretary, Ethics Committee, IRB
Additional Vice Principal (Research)

June 14, 2014

Dr. Rajeshwari. B
PG Registrar
Department of Transfusion Medicine and Immunohaematology
Christian Medical College, Vellore 632 004

Sub: **Fluid Research grant project:**
Serological characterization of antibodies in Autoimmune Hemolytic Anemia and its clinical implications -A study from tertiary care center in South India.
Dr. Rajeshwari. B, PG Registrar, Dr. Dolly Daniel, Dr. Mary Purna Chacko, ransfusion Medicine and Immunohaematology, Dr. Biju George, Haematology.

Ref: IRB Min No: 8902 [OBSERVE] dated 09.06.2014

Dear Dr. Rajeshwari. B,

The Institutional Review Board (Blue, Research and Ethics Committee) of the Christian Medical College, Vellore, reviewed and discussed your project entitled "To study the prevalence, burden, characteristics and prognostic significance of premature ventricular complexes (PVCs), as determined in 24 hour telemetric (holter) electrocardiographic recordings." on June 9th 2014.

I enclose the following documents:-

1. Institutional Review Board approval 2. Agreement

Could you please sign the agreement and send it to Dr. Nihal Thomas, Addl. Vice Principal (Research), so that the grant money can be released.

With best wishes,

Dr. Nihal Thomas
Secretary (Ethics Committee)
Institutional Review Board

Dr. NIHAL THOMAS
MD.,MNAMS.,DNB(Endo),FRACP(Endo),FRCP(Edin),FRCP(Glasg)
SECRETARY - (ETHICS COMMITTEE)
Institutional Review Board,
Christian Medical College, Vellore - 632 002.

Cc: Dr. Dolly Daniel, Transfusion Medicine and Immunohaematology, CMC, Vellore.

Ethics Committee Blue, Office of Research, 1st Floor, Carman Block, Christian Medical College, Vellore, Tamil Nadu 632 002.
Tel : 0416 - 2284294, 2284202 Fax : 0416 - 2262788, 2284481 E-mail : research@cmcvellore.ac.in

Dr. B.J. Prashantham, M.A., M.A., Dr. Min (Clinical)
Director, Christian Counseling Center,
Chairperson, Ethics Committee.

Dr. Alfred Job Daniel, D Ortho, MS Ortho, DNB Ortho
Chairperson, Research Committee & Principal

Dr. Nihal Thomas,
MD., MNAMS., DNB (Endo), FRACP (Endo), FRCP (Glas) (EDIN)
Deputy Chairperson
Secretary, Ethics Committee, IRB
Additional Vice Principal (Research)

We approve the project to be conducted as presented.

The Institutional Ethics Committee expects to be informed about the progress of the project, any **adverse events** occurring in the course of the project, any **amendments in the protocol and the patient information / informed consent**. On completion of the study you are expected to submit a copy of the **final report**. Respective forms can be downloaded from the following link: http://172.16.11.136/Research/IRB_Polices.html in the CMC Intranet and in the CMC website link address: http://www.cmch-vellore.edu/static/research/Index.html.

Fluid Grant Allocation:

A sum of 45,955/- INR (Rupees Forty Five Thousand Nine Hundred and Fifty Five only) will be granted for 18 months.

Yours sincerely

NOT TO BE MINISTERED UNTO BUT TO MINISTER
CHRISTIAN MEDICAL COLLEGE
VELLORE
INDIA

Dr. Nihal Thomas
Secretary (Ethics Committee)
Institutional Review Board

Dr. NIHAL THOMAS
MD.,MNAMS.,DNB(Endo),FRACP(Endo),FRCP(Edin),FRCP(Glasg)
SECRETARY - (ETHICS COMMITTEE)
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Tel : 0416 - 2284294, 2284202 Fax : 0416 - 2262788, 2284481 E-mail : research@cmcvellore.ac.in

Serological characterization of antibodies in Autoimmune Haemolytic Anemia and its clinical implications-A study from tertiary care center in South India

Clinical Research Form

Serial number	
Date	
Hospital no	
Age	
Gender	
Previous transfusions	

Severity of in vivo haemolysis

	Patient value	yes	No
Haemoglobin<9gm/dl			
Total Bilirubin >2mg/dl			
Reticulocytes>2%			
LDH>500IU/ml			

Primary AIHA – YES ☐ NO ☐

Secondary AIHA- YES ☐ NO ☐

If yes for secondary AIHA (√)If present

Lymphoproliferative disease		Infection	
Autoimmune disease		Others	

DAT TEST	**POSITIVE**	**NEGATIVE**	**Strength of DAT**	
DAT with Polyspecific antihuman globulin(AHG) and C3d				
DAT with monospecific IgG AHG				
DAT with monospecific IgA AHG				
DAT with monospecific IgM AHG				
DAT - C3c				
DAT-C3d				
DAT wih1:10 IgG				
DAT with monospecific IgG1 AHG			1:1 dilution	1:100 dilution
DAT with monospecific IgG3 AHG			1:1	1:100

Serological characterization of antibodies in Autoimmune Haemolytic Anemia and its clinical implications-A study from tertiary care center in South India

WBC		Neutrophils		Lymhocyte		Eosiphil		Basophil		Monocyte	
PLT count											
Direct bilirubin											
Albumin											
Total protein						Hospital no					
AST											
ALT											
Alkaline phosphatase											
ANA											
Ds DNA											
Complement											
Lupus/SLE											
Rash/joint pain											
Hair loss/organs involvement											
Peripheral smear											
BM result											
Hepatomegaly											
Splenomegaly											
Diagnosis											
Additional information											

Clinical data

Slno	Date	Age	sex	Pre trans	Hb	Hb value	bilir	Bil value	reti	Ret value	LDH	sple	hep	Peri smear	Dir bil
1	19/02/2015	70	1	1	1	6.6	1	2	1	3.8	1	0	0	1	1
2	05/08/2014	46	2	0	1	5.9	0	1.9	1	21.5	1	0	0	1	0.7
3	08/10/2014	18	2	1	1	6.1	1	5.9	1	15	1	1	0	1	0.6
4	06/10/2014	63	2	1	1	4.5	1	2.4	1	2.6	1	0	0	1	0.9
5	01/10/2014	34	2	0	1	5.5	1	4.5	1	38.9	1	1	0		1.5
6	26/09/2014	36	1	0	0	10.9	1	5.9	1	8	1			1	0.7
7	27/09/2014	1	1	0	1	2	1	2.6	1	9.2	1	0	0	1	0.7
8	07/10/2014	36	2	0	1	8.5	1	2.7	1	16.8	1	0	0	1	0.3
9	22/02/2014	20	2	0	0	9.7	0	0.4	1	2.8	1	0	0	2	0.1
10	17/10/2014	19	2	0	1	6.1	1	4.6	1	2.6	1	1	1	1	0.5
11	17/10/2014	52	2	0	1	3.6	0	1.8	1	18.3	1	0	0	1	0.5
12	27/10/2014	47	2	0	1	5.9	0	0.7	1	4.6	1	0	1	1	0.1
13	27/10/2014	35	2	0	1	7.7	0	0.4	1	9.5	1	0	0	1	0.2
14	24/10/2014	20	2	0	1	6.6	0	0.7	1	16.4	1	0	0	1	0.2
15	27/10/2014	25	2	0	1	7.1	1	11	1	6.8	1	0	0	1	1.2
16	10/02/2015	32	2	0	1	3.6	1	3.5	1	18	1	1	0	1	1.4
17	03/11/2014	53	1	1	1	4.1	1	32.9	1	15.8	1	1	1	1	25.8
18	06/11/2014	35	1	1	1	5.9	0	1.3	1	3	1	1	0	1	0.5
19	06/11/2014	41	1	0	1	7.3	1	12.4	1	4.3	0	1	0	1	7.4
20	12/11/2014	24	1	0	1	2.9	1	15.7	1	2.6	1	0	1	1	0.9
21	15/11/2014	45	1	0	1	3.6	1	16.1	1	24.9	1	0	0	1	2.6
22	18/11/2014	36	2	0	1	7.1	1	0.6	1	13.5	0	0	0	0	0.1
23	14/11/2014	45	2	1	1	6.5	1	6.7	1	45.2	1	1	1	1	1.1
24	20/11/2014	55	2	0	0	11.1	1	2.7	1	16.7	1	0	0	1	1.1
25	03/12/2014	31	1	0	1	4.8	1	8.7	0	0.2	1	0	0	1	0.9
26	05/12/2014	12	2	0	1	8.5	1	2.4	1	6.5	1	1	1	1	2
27	05/12/2014	48	1	1	1	6.9	1	3.3	1	7.5	1	1	0	1	0.5

Slno	Date	Age	sex	Pre trans	Hb	Hb value	bilir	Bil value	reti	Ret value	LDH	sple	hep	Peri smear	Dir bil
28	05/12/2014	1	1	0	0	13	0	0.5	1	7.2	1	0	0	1	0.3
29	09/12/2014	41	2	0	1	6.5	0	0.2	1	6.7	1	0	0	1	0.1
30	13/12/2014	35	1	0	1	5.3	1	5.8	1	8.8	1	1	0	1	2.6
31	15/12/2014	42	2	0	1	7.3	0	1.9	1	14.4	1	1	0	1	0.7
32	16/12/2014	46	2	0	1	7.2	1	3.2	1	22.5	1	1	0	1	0.8
33	17/12/2014	25	2	0	1	7.7	0	0.4	0	1	1	0	0	1	0.1
34	17/12/2014	47	2	1	1	7.1	0	1.8	0	1.7	1	0	1	0	0.4
35	28/12/2014	56	2	1	1	8.6	1	4.8	1	20.1	1	1	0	1	1.8
36	28/12/2014	47	2	0	1	5.5	1	3.8	1	6.2	1	0	0	1	1
37	05/09/2014	46	2	0	1	3.9	0	0.3	1	2.6	0	0	0	0	0.2
38	24/10/2014	20	2	0	1	6.3	0	0.7	1	10.7	1	0	0	1	0.2
39	31/10/2014	25	2	0	1	7.9	1	4.1	0	0.8	1	0	0	0	2.9
40	26/12/2014	13	1	0	1	4.9	1	3.2	1	21.5	1	0	0	1	0.7
41	26/12/2014	30	2	1	1	8.7	0	1.9	1	5	1	1	1	0	0.8
42	02/12/2014	22	1	0	1	2.6	0	1.2	1	6	1	0	0	1	0.5
43	25/12/2014	17	2	0	1	3	1	2.4	1	25.9	1	1	1	1	0.5
44	09/01/2014	19	2	0	1	7.5	0	0.4	1	2.9	1	1	0	0	0.1
45	09/01/2015	28	2	0	1	7.4	0	0.4	1	2.4	1	0	0	2	0.2
46	21/01/2015	59	1	1	1	5.6	1	3.8	1	2.8	1	0	0	1	0.8
47	17/01/2015	23	2	0	1	6.6	0	0.3	0		1	1	1	2	0.1
48	21/01/2015	19	1	1	1	6.5	1	6.2	1	15	1	1	1	1	1.5
49	04/02/2015	44	2	1	1	8.1	1	16.4	1	3.9	1	0	0	1	8.6
50	04/02/2015	62	2	0	1	8.6	0	0.7	1	7.9	1	1	0	1	0.8
51	04/02/2015	19	2	0	1	7.2	0	0.3	0	0.6	1	0	0	2	0.1
52	07/02/2015	61	2	0	0	9.9	0	1.2	1	3.9	1	0	0	1	0.4
53	20/02/2015	70	1	0	1	6.6	1	2	1	3.9	1	0	0	1	1
54	03/03/2015	47	2	1	1	5.3	1	8.9	1	23.4	1	1	0	1	1.8

Slno	Date	Age	sex	Pre trans	Hb	Hb value	bilir	Bil value	reti	Ret value	LDH	sple	hep	Peri smear	Dir bil
55	02/03/2015	12	1	0	1	8.4	1	3.1	1	5.9	1	1	0	1	0.6
56	03/03/2015	30	2	0	1	6.5	1	3.4	1	19.4	1	1	0	1	0.9
57	03/03/2015	48	2	0	1	6.4	1	2.9	1	12.9	1	1	0	1	1.3
58	04/03/2015	22	2	1	1	7.6	0	0.4	0	1.2	1	0	0	2	0.2
59	21/02/2015	20	2	0	0	9.4	0	0.4	1	2.8	1	0	0	2	0.1
60	03/03/2015	52	2	1	1	6.6	0	0.4	0	1.9	1	0	0	1	0.2
61	03/03/2015	34	2	0	1	7	0	0.4	1	2	1	0	1	2	0.3
62	18/03/2015	22	2	0	1	4.8	1	19.4	1	4.7	1	1	0	1	15.2
63	27/03/2015	52	1	0	0	10.8	0	0.5	1	2.5	1	1	0	1	0.2
64	30/03/2015	21	2	0	1	4.5	1	7.6	1	26.5	1	1	1	1	1
65	30/03/2015	54	1	0	1	7.4	1	10.2	1	6.9	1	1	1	1	0.9
66	24/03/2015	55	1	0	1	8.6	1	3	1	6.2	1	1	0	1	0.6
67	23/03/2015	24	2	0	0	10.4	0	0.6	1	2.8	1	0	0	0	0.3
68	23/03/2015	15	1	1	1	5.7	1	2	1	7.5	1	0	0	1	0.9
69	20/03/2015	48	1	0	1	7.8	1	1.3	1	14.2	1	1	1	0	0.6
70	19/03/2015	22	2	0	1	4.9	1	19.4	1	4.7	1	1	0	2	15.2
71	20/03/2015	48	1	0	1	7.8	0	1.3	1	14.2	1	1	0	1	0.6
72	23/03/2015	15	2	1	1	5.7	1	2	1	7.5	1	0	0	2	0.9
73	25/03/2015	24	2	0	0	10.2	0	0.6	1	2.4	1	0	0	2	0.3
74	25/03/2015	55	1	0	1	8.6	1	3	1	6.2	1	0	0	1	0.6
75	01/04/2015	54	1	0	1	7.6	1	10.2	1	7.9	1	1	1	1	0.9
76	01/04/2015	21	2	1	1	4.5	1	7.6	1	26.5	1	1	1	1	1
77	01/04/2015	52	1	0	0	10.8	0	0.5	1	2.5	1	0	0	2	0.2
78	08/04/2015	55	2	1	1	4.6	1	3	1	15.4	1	0	0	1	0.7
79	08/04/2015	27	2	0	1	8.7	1	3	1	15.4	1	0	0	1	0.9
80	21/04/2015	34	2	0	1	5.4	0	1.9	1	0.1	1	0	1	1	1.2
81	25/04/2015	39	2	1	1	6.1	0	1	1	11.5	1	1	1	1	0.5
82	28/04/2015	51	1	1	1	5	1	3.3	1	17	1	1	1	1	0.7

Slno	Date	Age	sex	Pre trans	Hb	Hb value	bilir	Bil value	reti	Ret value	LDH	sple	hep	Peri smear	Dir bil
83	30/04/2015	24	2	1	1	6.5	0	1.9	1	21.7	1	0	0	1	0.8
84	04/05/2015	40	2	0	1	6.4	0	0.5	1	5	1	1	0	2	0.1
85	06/05/2015	57	2	0	1	7.3	0	0.9	1	9.4	1	0	0	2	0.2
86	07/05/2015	48	2	1	1	3.5	1	5.8	1	41.4	1	1	1	1	0.9
87	06/05/2015	50	1	0	1	5.6	0	1.9	1	15.3	1	1	1	1	0.5
88	25/12/2015	17	2	0	1	3	1	2.4	1	25.7	1	1	1	1	0.5
89	20/12/2015	49	1	1	1	3.7	1	8	1	3	1	0	0	1	0.3
90	22/12/2015	26	2	0	1	5.5	1	0.5	1	2.2	1	0	0	1	0.2
91	26/12/2015	13	1	0	1	4.9	1	3.2	1	21.5	1	0	0	1	0.7
92	16/05/2015	34	2	0	1	4.7	1	2.3	1	20.5	1			1	0.5
93	26/12/2014	30	2	1	1	3.6	0	1.9	1	5	1	1	1	1	0.8
94	20/05/2015	77	1	0	1	6.3	1	3.7	1	19.3	1	0	0	1	0.7

Bone marrow	primary	secondary	causes	auto	other	diag	steroids	medication	Poly DAT
2	0	1	9		mds and lymphoma	1	1	no	3
	1	0				1	0	no	3
1	1	0				1	0	4 units blood	4
1	1	0				1	0	no	4
	1	0				1	0	no	4
	1	0				0	0	azoran 50mg	4
1	1	0				1	0	no	4
	1	0				0	1	azoran 100mg	4
2	0	1	2	4	nephrotic syndrome	0	1	no	3
	0	1	2	4	evans syndrome	1	0	no	4
2	1	0				1	0	no	4

Bone marrow	primary	secondary	causes	auto	other	diag	steroids	medication	Poly DAT
1	0	1	2			1	0	no	4
9	0	1	2	1	cns lupus	1	0	no	2
1	0	1	9		cold agglutinin titre positive	1	0	no	4
	1	0				0	1	no	4
9	1	0				1	0	blood transfusion	4
2	0	1	9		homeopathy medicine intake	1	0	2 blood transfusion	4
0	1					0	1	2 blood transfused	4
0	0	1	9		ethanol intake	1	0	no	2
1	1	0				1	0	no	4
0	1					0	0	no	3
0	0	1	2	4	proteinuria	1	0	n0	4
0	1					0	0	no	4
2	1					0	1	no	4
2	1					1	0	no	4
0	0	1	2	2	serositis	0	1	no	4
0	0	1	3		mycoplasma titre +	0	0	no	2
0	0	1	1		alps	0	1	no	4
9	0	1	2	4	collagen vascular disease + gn	0	1	cyclophosphamide	2
1	1	0				0	0	no	2
1	1					0	1	no	2
0	1	0				1	0	no	4
2	0	1	2	4	autoimmune hepatitis	1	0	no	2
9	0	0	1		lymphoma	1	0	no	2
1	1	0				1	0	no	4
0	1	0				0	0	azoran 50 mg	4
2	1					1	0	no	4

Bone marrow	primary	secondary	causes	auto	other	diag	steroids	medication	Poly DAT
1	0	1	3	4	mixed connective d, mycoplasma	1	0	no	3
9	0	1	2	1	joint pain, organ involvement	1	0	no	3
1	1					1	0	no	3
1	0	1	1		lymphoma	1	0	blood transfusion	3
0	1					1	0	no	4
2	0	1	2	4	evans	0	1	no	3
9	0	1	2	1	joint pain	0	1	azoran 75 mg	2
0	0	1	2	1	joint pain,sle	1	0	no	4
1	1					1	0	no	4
0	0	1	2	1	nephrotic syndrome	1	0	no	4
2	0	1	9		myelofibrosis	0	1	cyclosporine 150 mg	3
0	0	1	2			0	1	azoran 100 mg	4
0	1					0	1	danazol	4
9	0	1	2	3	serositis	1	0	no	4
2	0	1	1		cryoglobulinaemia	0	1	azoran 250 mg	4
2	0	1	9		mds	0	1	no	3
1	1	0				1	0	no	4
0	0	1	2			1	0	no	4
0	1					0	1	no	4
0	1					0	1	mmf 250 mg bd	4
1	0	1	2	1	nephritis,hair loss	1	0	no	4
0	0	1	2	4	lupus nephritis	1	0	no	3
9	0	1	2	4	lupus nephritis	1	0	no	2
0	0	1	2	4	nephritis	0	1	n0	4
1	0	1	2	4	nephritis	1	0	no	4
1	1	0				0	0	no	3

Bone marrow	primary	secondary	causes	auto	other	diag	steroids	medication	Poly DAT
1	1	0				0	1	no	4
0	1	0				1	0	n0	4
0	1	0				0	1	no	4
9	0	1	2	1	nephritis	1	0	n0	3
1	1	0				1	0	no	4
2	0	1	1			1	0	no	4
2	0	1	2	2		0	0	no	4
2	0	1	1			1	0	no	4
0	1					1	0	n0	4
9	0	1	2	4	lupus nephritis	1	0	n0	3
0	1	0				0	1	no	3
0	1					1	0	n0	4
1	1	0				0	1	no	4
1	1	0				0	0	blood transfusion	4
1	1	0				1	0	n0	4
2	1	0				0	1	cyclosporin azoran	4
2	0	1	2			0	0	n0	4
0	1	0				0	1	no	4
1	1	0				0	0	n0	4
0	1	0				1	0	n0	4
1						1	0	n0	3
0	1					1	0	n0	4
1	1					1	0	no	4
1	1					1	0	n0	4
1	0	1	2	4		1	0	n0	4
2	0	1	9		mutiple myeloma	1	0	no	2
1	0	1	2	4	nephritis	1	0	n0	2
1	1	0							4
2	1	0							4
1	1	0				1	0	blood transfusion	3
	1	0							4

MonoIgG	+IgG	MonoIgA	+IgA	MonoIgM	+IgM	datc3c	+c3c	datc3d	+c3d	IgG1:10
1	3	0		0		0		1	3	1
1	2	0		0		0		0		1
1	4	0		1	4	1	4	1	4	1
1	4	0		0		0		1	4	1
1	3	0		0		0		1	4	1
1	4	1	4	0		0		0		1
1	4	0		0		0		0		1
1	4	0		0		0		0		1
1	3	0		0		0		0		1
1	4	0		1	3	0		1	4	1
1	4	0		1	4	0		1	4	1
1	4	0		0		0		1	3	1
1	3	0		0		0		0		1
1	4	0		1	3	1	3	1	4	1
1	4	0		0		0		1	3	1
1	4	0		0		0		1	4	1
1	4	0		0		0		1	4	1
1	4	0		0		0		1	3	1
1	2	0		0		0		0		1
1	4	0		1	3	1		1		1
1	3	0		0		0		1	3	0
1	4	0		0		0		1	4	1
1	4	1	3	1	3	0		1	4	1
1	4	0		0		0		1	3	1
1	4	0		0		0		0		1
1	4	0		0		0		0		1
0		0		0		0		1	3	0
1	4	0		0		0		0		1

MonoIgG	+IgG	MonoIgA	+IgA	MonoIgM	+IgM	datc3c	+c3c	datc3d	+c3d	IgG1:10
1	2	0		0		0		0		0
0		0		0		0		1	4	0
1	2	0		0		0		1	2	0
1	4	0		0		0		1	3	1
1	2	0		0		0		0		1
0		0		0		0		1	2	0
1	4	0		0		0		0		1
1	3	0		0		0		1	3	1
1	4	0		0		0		0		1
1	4	0		1		1		1		1
1	3	0		0		0		0		1
1	4	0		1	3	1	3	1	4	1
1	4	0		1	2	0		1	2	1
1	4	1	3	0		1	1	1	4	1
1	4	0		1	3	0		1	3	1
1	2	0		0		0		0		0
1	3	0		0		0		0		1
1	4	0		0		0		1	3	1
1	3	0		0		0		0		1
1	1	0		0		0		1	1	0
1	4	0		1	2	0		0		1
1	4	0		0		0		1	3	1
1	4	0		0		0		0		1
0		0		0		0		1	4	0
1	3	0		0		0		1	3	1
1	4	0		0		0		1	4	1
1	4	0		1	3	0		1	4	1

MonoIgG	+IgG	MonoIgA	+IgA	MonoIgM	+IgM	datc3c	+c3c	datc3d	+c3d	IgG1:10
1	4	0		1	2	0		1	4	1
1	4	0		1	2	1	2	1	4	1
1	4	0		1	3	0		1	3	1
1	4	0		0		0		0		1
1	2	0		0		0		0		0
1	4	0		1	4	0		1	4	1
1	4	0		0		0		1	4	1
1	4	0		0		0		1	3	1
1	4	0		0		0		1	4	1
0		0		0		0		1	4	0
0		0		0		0		1	4	0
1	3	0		0		0		0		1
1	4	0		1	4	0		1	4	1
1	4	0		0		0		0		1
1	4	0		0		0		1	4	1
1	4	0		0		0		0		1
1	4	0		1	4	0		1	4	1
1	3	0		0		0		0		1
0		0		0		0		1	4	0
0		0		0		0		1	4	0
1	4	0		0		0		1	4	1
1	4	0		0		0		1	3	1
1	4	1	1	0		0		1	3	1
1	4	0		0		0		1	1	1
1	4	0		1	3	0		1	4	1
1	4	0		0		0		1	4	1
1	4	0		1	4	1	4	1	4	1
1	4	0		0		0		1	4	1
1	3	0		0		0		0		1
1	4	0		0		0		1	3	1

MonoIgG	+IgG	MonoIgA	+IgA	MonoIgM	+IgM	datc3c	+c3c	datc3d	+c3d	IgG1:10
1	4	0		1	3	0		1	3	1
1	4	1	2	1	1	0		0		1
1	4	0		1	4	0		1	4	1
1	2	0		0		0		0		0
1	3	0		0		0		0		1
1	4	0		1	3	1	4	1	4	1
1	4	1	2	1	3	0		1	4	1
1	4	0		1	3	0		1	3	1
1	4	0		1	3	0		1	3	1

+1:10	IgG1	+1:1	+1:100	IgG3	+1:1	+1:100	severity
3	0			0			1
2	0			0			2
4	1	4	1	0			1
3	1	3	0	0			1
2	0			0			1
4	1	4	4	0			1
4	1	4	3	0			1
4	1	4	2	0			1
3	0			0			2
3	1	2	0	0			1
3	1	2	0	0			2
3	1	2	0	0			2
2	0			0			2
3	1	2	0	0			2
4	1	4	2	1	3	1	1
4	1	2	2	0			1

+1:10	IgG1	+1:1	+1:100	IgG3	+1:1	+1:100	severity
4	1	4	3	0			1
4	1	4	3	0			2
2	0			0			2
4	1	4	4	1	3	0	1
	0			0			1
4	1	4	0	0			1
4	1	4	4	0			1
4	1	4	1	0			2
4	1	3	0	0			2
4	1	4	3	0			1
	0			0			1
4	1	4	0	1	4	3	2
	0			0			2
	0			0			1
	0			0			2
4	1	4	3	0			1
1	0			0			2
	0			0			2
4	1	4	3	0			1
3	1	2	0	0			1
3	0			0			2
3	1	2	0	0			2
2	0			0			2
4	1	4	2	0			1
4	1	4	1	1	4	3	2
4	1	4	3	0			2
3	1	2	0	0			1

+1:10	IgG1	+1:1	+1:100	IgG3	+1:1	+1:100	severity
	0			0			2
3	0			0			2
3	1	3	0	0			1
3	1	2	0	0			2
	0			0			1
3	0			0			1
3	1	3	0	0			2
4	1	3	0	0			2
	0			0			2
3	0			0			1
4	1	4	3	1	4	1	1
3	1	1	0	0			1
3	1	3	0	0			1
4	1	4	3	0			1
4	1	4	3	0			2
3	0			0			2
	0			0			2
3	1	2	0	0			2
3	0			0			1
4	1	4	0	0			2
4	1	4	0	0			1
	0			0			1
	0			0			1
2	0			0			2
4	1	4	3	0			1
4	1	4	0	0			1
3	0			0			1
4	1	4	0	0			2

+1:10	IgG1	+1:1	+1:100	IgG3	+1:1	+1:100	severity
4	1	4	3	0			1
1	0			0			2
	0			0			1
	0			0			1
4	1	4	0	0			1
4	1	4	0	0			2
4	1	4	3	0			1
4	1	1	0	0			1
4	1	4	0	0			2
3	1	4	0	0			2
4	1	4	0	0			1
4	1	4	1	1	4	1	1
3	0			0			2
4	1	4	1	0			2
4	1	4	4	1	4	3	1
4	1	4	0	1	1	0	2
3	1	3	0	0			1
	0			0			2
2	0			0			2
4	1	4	2	0			1
4	1	4	3	1	3	0	1
4	1	4	0	1	4	3	1
4	1	4	3	0			1